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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07J 9/00, A61K 31/575 | A1

(11) International Publication Number: WO 97/45440

(43) International Publication Date: 4 December 1997 (04.12.97)

(21) International Application Number: PCT/SE97/00936

(22) International Filing Date: 29 May 1997 (29.05.97)

(30) Priority Data:
9602100-1 30 May 1996 (30.05.96) SE

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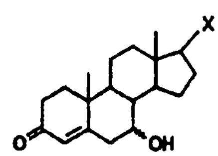
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

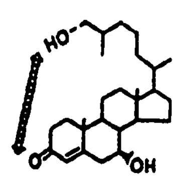
(54) Title: CYTOSTATIC STEROLS

(57) Abstract

Compounds of formula (I) wherein X is a straight or branched, hydroxy substituted $C_1$-$C_{15}$ hydrocarbon chain, have utility in the treatment of conditions associated with rapidly growing cells and cancers such as melanoma.



(I)



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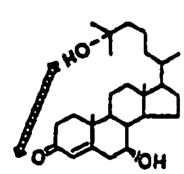

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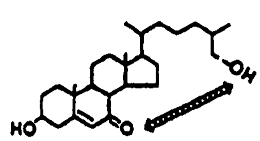

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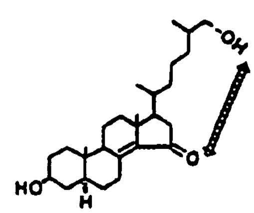
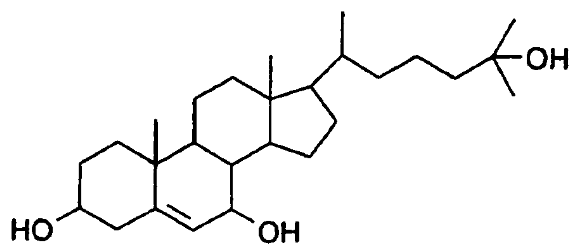


IV

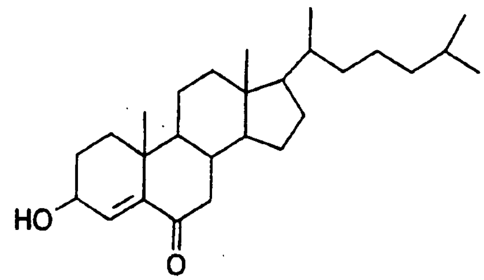
pp75-83. International patent application no. WO 91 11452 describes glycosylation of such compounds at the 3β-hydroxy and Rong et al C. R. Acad. Sc. Paris 1985, 300 Serie III, 89-94 describes the corresponding bis-hemisuccinate.

Moog et al in Biochimie 1991 73 (10) pp 1321-6 investigate the immunosuppressive effects of the $\Delta^5$ compound 7,25-dihydroxycholesterol:



which strongly inhibits lymphocyte response to different stimuli. Activity appeared to be related to protein kinase C and the incorporation of the dihydroxycholesterol into the cell membrane. Apparently the free $\Delta^5$ oxysterols do not depress tumour growth in living animals but Moog et al in Anticancer Res 13 (4) 953-8 (1993) and Ji, Moog et al, Canc. Biochem. Biophys 11 (1) 45-47 (1990) report that the phosphate diester of 7β,25-dihydroxycholesterol with a pyrimidine nucleoside is usable in a cytotoxic role. However Ji's observation that similar nucleoside-cholesterol esters act at the level of nucleic acid synthesis make the contribution to cytotoxicity played by the steroid viz a viz the nucleoside difficult to determine.

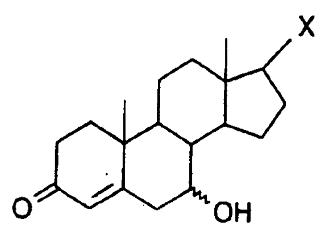
Analogues of the $\Delta^5$ compound 7β-hydroxycholesterol with branched, unsaturated side chains are implicated in rat hepatocyte toxicity at high (33 μg/ml) concentrations by Nagano et al, J Chem Res (S) 1977, 218, as are certain hydroxylated, unsaturated side chains in $\Delta^5$ analogues lacking the 7-hydroxy group. This work also describes the $\Delta^4$ compound:



where the double bonds are in different rings of the sterol possessed modest cytostatic activity at the relatively high concentration of 33 μg/ml.

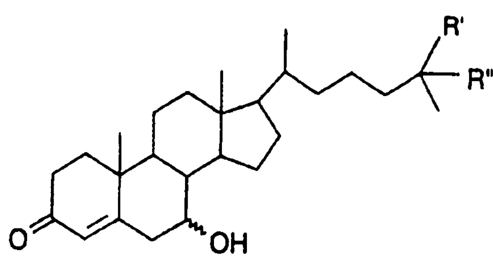
We have now discovered that compounds with a 3-keto group and conjugated double bond at $\Delta^4$ in conjunction with an hydroxylated side chain have cytostatic activities which are many times more potent than the compounds of the prior art.

In accordance with a first aspect of the invention there is provided compounds of the formula I:



wherein X is a straight or branched, hydroxy substituted $C_1$-$C_{15}$ hydrocarbon chain, for use in medicine.

A preferred group of compounds within the scope of the invention has the formula IA:



wherein one of R' and R"is OH and the other is H. Particularly preferred compounds of Formula I include 7β,25-dihydroxy-4-cholesten-3-one, 7α,25-dihydroxy-4-cholesten-3-one and 7β,27-dihydroxy-4-cholesten-3-one.

The enantiomer 7β,25-dihydroxy-4-cholesten-3-one does not appear to have been previously described in the literature and thus a further aspect of the invention provides this compound, preferably in substantially pure form, for instance > 75%, preferably > 90% and most preferably > 95% enantiomerically pure.

The invention further provides pharmaceutical compositions comprising the compounds of the invention, preferably those within Formula IA, in admixture with a physiologically acceptable diluent or pharmaceutical carrier.

Advantageous aspects of the invention include the use of the above group of compounds in the preparation of a medicament for the treatment of conditions associated with rapidly growing cells, such as various cancers and virus transformed mammalian cells. Representative cancers (which may or may not be virally transformed) include sarcomas, such as soft tissue sarcoma, myeloproliferative tumours such as leukaemia, glioblastoma, pancreatic, ovarian and adenocystic cancers. A further area of application is psoriasis, a disorder in which there is a loss of control of normal epidermal turnover. Increased mitosis of epidermal cells results in thickening of the epidermis and the production of imperfect keratin scales.

Thus the invention also provides a method for the treatment of conditions associated with rapidly growing cells comprising the administration of an effective amount of a compound of formula I, or more preferably Formula IA to a human or animal in need thereof.

A preferred aspect of the invention provides the above compounds for use in the preparation of a medicament or in methods for the treatment of breast carcinoma or colonic carcinoma in a human or animal. A particularly preferred aspect of the

invention provides use of the above groups of compounds for use in the preparation of a medicament or in methods for the treatment of malignant melanoma cells.

The compounds of the invention include the corresponding pharmaceutically accceptable derivatives as are known in the steroid art and which release the respective compound of Formula I in vivo. Suitable derivatives include ethers and esters of the hydroxy groups and/or derivatives of the oxo group. Representative esters and ethers include glycosides, such as the hexoses described in the abovementioned WO 91 11452, and bis-hemisuccinates, phosphates, glycoside phosphates, silyl ethers, acetate, formate and other fatty esters, such as the oleate, glucuronides, phosphodiesters, cyclodextrins, such as 2-hydroxy-β-cyclodextrin and the like as are known in the steroid art. Other derivatives include the corresponding oximes and pharmaceutically acceptable salts.

As shown in greater detail below, the compounds of the invention are subject to intracellular metabolism and such active metabolites are within the scope of the invention. For instance, 7,25-dihydroxycholest-4-en-3-ones may be metabolized to the corresponding cholestenoic acids. The administration to a mamal, including humans, of such active metabolites for the indications specified above is thus to be regarded as an aspect of the invention.

The nature of the pharmaceutical preparation of the invention will depend on the disease being treated and the examples below are not intended to be limiting.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, troches, powders, solution, suspensions, or emulsions.

For parenteral administration, the compounds may be administered as injectable dosages of a solution, suspension or emulsion of the compound in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as

water, alcohols, oils and other acceptable organic solvents, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants.

The compounds can also be administered in the form of a depot injection or implant preparation which may be formulated in such manner as to permit a sustained release of the active ingredient.

For topical application the compounds can be administered in the form of an unguent, cream, ointment, or a lotion.

The invention will be further described below by way of example only with reference to the enclosed Figures, in which:

Fig. 1 outlines a simplified scheme of the metabolism of LDL (low density lipoprotein) cholesterol and major autooxidation products of fibroblasts and the formation of potent HMG-CoA reductase suppressors. The names of the steroids are listed in Table I. In addition to hydrolysis and esterification (not shown) major reactions of sterols are I, 27-hydroxylation, II, 7α-hydroxylation; III, 3-oxidation with isomerization of the 5-double bond; and IV, oxidation to a 27-carboxy group. Hydrolyzed LDL cholesterol is metabolized via these reactions (filled arrows). Oxidation of a 7β-hydroxy group (V) to a ketone is also observed. Minor reactions noted are shown by broken arrows. The formation of 7α,25-dihydroxy-4-cholesten-3-one by 25-hydroxylation of sterols (not shown) was observed under specific conditions. Reactions I, II and III were obstructed in virus-transformed fibroblasts displaying a defective suppression of HMG-CoA reductase by LDL cholesterol and autooxidation products of cholesterol. Sterol metabolites with an apparently normal suppressive effect also in transformed cells are indicated in frame.

Fig. 2 shows structures of the three naturally occurring cholesterol derivatives that were potent suppressors of HMG-CoA reductase also in transformed fibroblasts. The sterols 7α,27-dihydroxy-4-cholesten-3-one (I), 7α,25-dihydroxy-4-cholesten-3-one (II), and 27-hydroxy-7-oxo-cholesterol (III) did not seem to require further

metabolism in order to suppress HMG-CoA reductase in human fibroblasts. Common to these sterols is the presence of an oxo group with a conjugated double bond in the steroid nucleus and a distal hydroxyl group in the side chain. The sterols are drawn in such a way that their apparent structural similarities are illustrated. For comparison, 27-hydroxylated 3β-hydroxy-5α-cholest-8(14)-en-15-one (IV) is also shown.

Fig. 3 discloses gas chromatographic-mass spectrometic GC/MSanalysis of neutral oxysterols isolated from the medium after incubating normal fibroblasts with lipoproteins. Normal human fibroblasts (protein contents 1.1 mg, dish size: 143 $cm^2$) were incubated with 10 ml of medium containing 10 % FCS (fetal calf serum); (cholesterol concentration: 1,2 mM) for 24 h, and the medium was then taken for analysis by GG/MS. Fragment ion current chromatograms characteristic of the trimethylsilyl ethers of oxysterols were constructed by the computer from mass spectra taken every 2h during the analysis and for the pupose of illustration the intensities of the ions (m/z) were multiplied by appropriate factors. The principal sterols indicated by the numbers are listed in Table I. The equivalent of about 0.2 ml of medium was injected onto a Finnigan SSQ 710 instrument housing a 25-m fused-silica column coated with methyl silicone, and the oven temperature was programmed from 185 till 280°C at a rate of 5°C x min $^{-1}$.

Fig. 4 relates to HPLC analyses of $^{3}$H-labeled 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenoic acid isolated from the medium of normal human fibroblasts. The cells (0.7 mg of protein) were incubated for 68 h with LDL (4% FCS) labeled with [$^{3}$H]cholesteryl oleate, and the medium was then taken for analysis by HPLC. For experimental details, see "General Methods" hereafter and Table IV. After the injections, fractions of the HPLC column effluent were collected in scintillation vials with 15-60 s intervals, and the radioactivity was then determined. For purpose of illustration, unlabeled 7α,27-dihydroxy-4-cholesten-3-one (results shown in top chromatograms) or 7α-hydroxy-3-oxo-4-cholestenoic acid (as methyl ester derivative, bottom chromatograms) were injected together with the products, and the peaks of these compounds are seen in the UV chromatograms. A

column of silica (LiChrosper) connected to a UV detector was used with hexane/isopropyl alcohol (90:10) as mobile phase, and the flow rate was 1.0 ml x $min^{-1}$.

Fig 5. shows effects of LDL on HMG-CoA reductase in normal (O) and virustransformed (●) human fibroblasts. Activities of HMG-CoA reductase in the fibroblasts were determined after incubation for 24 h with media containing diffferent concentrations of FCS. In the absence of FCS, media contained 10% LDS (lipoprotein deficient serum). All cells were preincubated for 24 h in media containing 10% LDS. The concentrations of cholesterol in FCS and LDS were 1.2 and 0.1 mM, respectively. The control activities of HMG-CoA reductase in normal and transformed cells were 72% and 101 pmol/min/mg of protein, respectively.

Fig. 6 discloses time-response curves for the LDL-induced production of 7α,27-dihydroxy-4-cholesten-3-one (▲), 27-hydroxy-7-oxocholesterol (●), and 7α,25-dihydroxy-4-cholestn-3-one (■), and suppression of HMG-CoA reductase (O) in normal human fibroblasts. The cells (protein content: 1.7 mg, dish size: 143 $cm^2$) were incubated with medium (15 ml) containing 10% FCS and were harvested at the indicated times. The concentration of cholesterol in FCS was 1.2 mM. All cells were preincubated for 24 h in media containing 10% LDS (see also Table III). For comparison, the production of 27-hydroxycholesterol (Δ) is also shown.

The invention will now be illustrated by way of example only with reference to the following non-limiting examples.

General methods

The steroids used were obtained in the following way: Diosgenin ((25R)-5-spirosten -3β-ol) was from Sigma (USA) and was used as the starting material for the synthesis of 27-oxygenated steroids (Arunachalam et al, (1981) J. Org. Chem. vol. 46, 2966-2968; Fieser et al., (1967) Reagents for Organic Synthesis, p. 1059, John Wiley and Sons Inc., New York; Shoda et al., (1993) Steroids, vol. 58, 119-125). In

addition, 5-cholestene-3β,7α,25-triol was prepared from the 3-acetate,25-trimethylsilyl ether derivative of 25-hydroxycholesterol, and after hydrolysis, this steroid was further oxidized to 7α,25-dihydroxy-4-cholesten-3-one as described for the corresponding 27-hydroxysteroids (Shoda et al., supra). 25-Hydroxycholesterol was oxidized in the same way to 25-hydroxy-4-cholesten-3-one. The other steroids were those used in a previous study (Axelson et al (1995) J. Lipid Res vol. 36, 290-298) and we refer to this article as a disclosure on how to obtain these compounds.

Normal human fibroblasts (line GM 08333) were obtained from NiGMS, Corriell Institute for Medical Research (Camden, NJ) and SV40 virus-transformed human fibroblasts 90-VA IV) were a kind gift from Dr. Stein (University of Colorado, Boulder, CO). Human colonic carcinoma (WiDr), breast carcinoma (MDA 231), and malignant melanoma (SK-MEL-2) cell lines were from American Type Culture Collection.

Cell lines were grown in monolayers in tissue culture flasks maintained in a 95% air, 5% $CO_2$ atmosphere at 37°C in humidified incubator and were cultured in either Dulbecco´s Modified Eagle's Medium (MDA 213) or Minimal Eagle's Medium (the other cells) supplemented with essential and non-essential amino acids and 10% FCS (v/v). For experimental purposes, cells were cultured in dishes. Cells were seeded at a density of 5,000 cells per $cm^2$. The experiments were started 48-72h later, at which time a cell density of approximately 20,000 per $cm^2$ had been reached. When the metabolism of cholesterol or oxysterols was studied, normal or transformed fibroblasts (cell number 3-6 x $10^6$ in 57-143 $cm^2$ dishes) were first preincubated for 24 h in medium containing 10% LDS and were then incubated for 3-68 h with 7-10 ml of medium containing 4-10% FCS (with our without $^3$H-labeled cholesterol or cholsteryl oleate) or were incubated with the oxysterol in 10% LDS for 24-48 h. Control cells were incubated in the same way, but only for 15 min. Effects of cyclosporin A (CsA), ketoconazole, and oxysterols were tested on normal and transformed fibroblasts at concentrations of 10-30 μM, 30 μM, and 0.12 μM, respectively, in cell media containing 0-10% FCS and 10-0% LDS. The substances were added to the incubation media in freshly prepared ethanol solutions, and the

ethanol concentrations of media became 0.1-0.5%. Control cells were incubated in the same way, but without CsA, ketoconazole, or oxysterols. The dish size and volume of media when HMG-CoA reductase activity was to be determined were 20 $cm^2$ and 5 ml, respectively, and the incubations were carried out in duplicate for 3-24 h. Each oxysterol was tested in 2-5 separate experiments. Determination of HMG-CoA reductase activity was then carried out as described previously (Axelson et al (1995) J. Biol. Chem. <u>270</u>, 15102, Cavenee et al. (1981) J. Biol. Chem. <u>256</u>, 2675 and Edwards, P.A. et al. (1979) J. Lipids. Res. <u>20</u>, 40.

The procedure for extraction and purification of oxysterols present in incubation media and cells was essentially the same as described previously (Axelson 1995 supra). Following the collection of a neutral oxysterol fraction from the lipophilic anion exchanger a fraction containing steroids with a free carboxyl group was eluted with 0.15 M acetic acid in 95% aqueous methanol prior to elution of a fraction containing stronger acids (including steroid sulfates) with 0.5 M potassium acetate/potassium hydroxide, apparent pH 10.0 in 72% aqueous methanol (Axelson et al. J. Biol. Chem. (1991) <u>266</u>, 17770).

Trimethylsilyl ethers of oxysterols and methyl ester trimethylsilyl ether derivatives of steroid acids were prepared (Axelson 1991 supra) and were analyzed by gas chromatography mass spectrometry (GC/MS) as described in Axelson 1995 supra.

$^3$H-Labeled cholesterol, cholesteryl oleate, and 25-hydroxycholesterol and/or their radioactive metabolites were analysed by HPLC prior to or after group fractionation and purification as described above. $^3$H-labeled cholesterol and cholesteryl esters were extracted from small aliquots of the incubation media with mixtures of isopropyl alcohol and hexane and from cells with mixtures of ethanol and water prior to separation by straight-phase HPLC using hexane/isopropyl alcohol, 98:2 (v/v), as the mobile phase (Axelson 1995 supra). Appropriate fractions from the HPLC effluent were collected in vials, and the radioactivity was then determined by scintillation counting. Radioactive neutral and acidic metabolites of [$^3$H]cholesterol or [$^3$H]cholesteryl oleate were isolated from media and cells as described above and

were then characterized by HPLC: For this purpose, three HPLC systems were used in the following order. Reversed phase HPLC was carried out on a column of LiChrospher (250 x 4 mm, Hibar, 100RP-18, 5 μm, Merck, Darmstadt, Germany) using a pump (Constametric III) and a variable wavelength detector (Spectra Monitor D from LDC/Miton Roy, Riviera Beach, FL) set at 220 or 240 nm and a Rheodyne Model 7125 injector with a 100 μl loop. The mobile phase used for neutral metabolites was a mixture of methanol/ethanol/water, 80:20:10 (by volume, flow rate 1 ml x $min^{-1}$), and fractions were collected between 0 and 11 min (fraction 1; containing polar metabolites, e.g. 7α,27-dihydroxy-4-cholesten-3-one, retention time about 4.5 min) and between 11 and 14 min (fraction 2; containing 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol having retention times 11.5 and 12.5 min, respectively). The mobile phase was then changed to 85% aqueous methanol (flow rate 1 ml x $min^{-1}$) for separation of sterols in fraction 1 or for separation of steroid acids (as methyl ester derivatives). In the former case, a fraction of the effluent containing 7α,27-dihydroxy-4-cholesten-3-one (retention time about 8.5 min) was collected between 8.0 and 9.0 min, and in the latter case a fraction of the effluent containing 7α-hydroxy-3-oxo-4-cholestenoic acid (retention time about 12 min) was collected between 11.0 and 13.0 min. These fractions and fraction 2 (containing 7α-hydroxy-4-cholesten-3-one and 27-hydroxy-cholesterol) were then reanalyzed by straight-phase HPLC. The latter was carried out with an instrument similar to that above, but with a column (250 x 4.5 mm) of LiChrosper (Hibar, Si 100, 5 μM, Merck). The mobile phase was hexane/isopropyl alcohol, 94:6 (v/v), when fraction 2 was analyzed (retention times of 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol were about 8 and 9 min, respectively), and 90:10 (v/v), when the fractions containing 7α,27-dihydroxy-4-cholesten-3-one or 7α-hydroxy-3-oxo-4-cholestenoic acid methyl ester were analyzed. The flow rate was 1.0 ml x $min^{-1}$ in all cases. The HPLC effluent during the latter analyses was collected in scintillation vials with 15-60-s intervals, and after addition of scintillation fluid, the radioactivity was determined.

25-[$^3$H]Hydroxycholesterol and its metabolites in media and cells were analyzed by HPLC following extraction. Medium was extracted with ethanol, and, after

centrifugation and removal of the supernatant, the pellet was re-extracted with ethanol/isopropyl alcohol, 1:1 (v/v). The extracts were combined and the solvent was then evaporated. Nonpolar compounds were dissolved in hexane, and, after removal, the solid residue was dissolved in 60% aqueous methanol, which was passed through a column (1.5 x 0.8 cm) of octadecylsilane-bonded silica (Preparative $C_{18}$; Walters Associates Inc., Milford, MA) and collected. The methanol in the eluate was then removed in vacuo, and the aqueous solution was re-extracted on the same column. After washing the column with water, adsorbed steroids (polar metabolites) were eluted with methanol/chloroform, 1:1 (v/v), and were combined with the nonpolar metabolites present in the hexane fraction. This combined extract was evaporated to dryness and dissolved in methanol or hexane/isopropyl alcohol, 90:10 (v/v), prior to analysis by reversed-phase HPLC (mobile phase; methanol/ethanol/water, 80:20:10 (v/v) or straight-phase HPLC (mobile phase: hexane/isopropyl alcohol, 97:3 (v/v).

<u>Example 1</u>

<u>Formation of oxygenated cholesterol derivatives in human fibroblasts</u>

Table I shows the oxygenated cholesterol derivatives identified in the neutral and acidic fractions from media (containing 10% FCS) after incubation with normal human fibroblasts and their gas chromatographic/mass spectrometric characteristics as trimethylsilyl ethers and methyl ester trimethylsilyl ether derivatives, respectively.

| TABLE I<br>Steroid name | Structure[a] | Fig 3[e] | RI[b] | Molecular & significant ions[c] |
|---|---|---|---|---|
| Neutral $C_{27}$ Steroids | | | | *m/z* |
| 7α-Hydroxycholesterol | $C^5$-3β,7α-ol | 1 | 3115 | 546, *456* |
| 7α-Hydroxy-4-cholesten-3-one | $C^4$-7α-ol-3one | 2 | 3210 | 472,457,*382*,269 |
| 7β-Hydroxycholesterol | $C^5$-3β,7β-ol | 3 | 3235 | 546,*456* |
| 7-Oxocholesterol | $C^5$-3β-ol-7-one | 4 | 3375 | *472*,382,367,129 |
| 24-Hydroxycholesterol | $C^5$3β,24 (R/S)ol | 5 | 3385 | 546,*413*,145,129 |
| 25-Hydroxycholesterol | $C^5$-3β,25-ol | 6 | 3405 | 546,456,*131* |
| 7α,25-Dihydroxycholesterol | $C^5$-3β,7α-25-ol | 7 | 3390 | 634,*544*,131 |
| 7α,25-Dihydroxy-4-cholesten-3-one | $C^4$-7α,25-ol-3-one | 8 | 3490 | 560,545,412,*131* |
| 27-Hydroxycholesterol | $C^5$-3β,27-ol | 9 | 3455 | 546,*456*,417,129 |
| 7α,27-Dihydroxycholesterol | $C^5$-3β,7α,27ol | 10 | 3445 | 634,*544* |
| 7α,27-Dihydroxy-4-cholesten-3-one | $C^4$-7α,27-ol-3-one | 11 | 3545 | 560,545,*470*,269 |
| 7β,27-Dihydroxycholesterol | $C^5$-3β,7β,27ol | 12 | 3555 | 634,*544* |
| 27-Hydroxy-7-oxo-cholesterol | $C^5$-3β,27-ol-7-one | 13 | 3710 | *560*,545,470,129 |
| $C_{27}$-steroid acids | | | | |
| 3β-hydroxy-5-cholestenoate | $CA^5$-3β-ol | 14 | 3425 | 502,*412*,373,129 |
| 3β,7α-Dihydroxy-5-cholestenoate | $CA^5$-3β,7α-ol | 15 | 3415 | 590,*500* |
| 7α-Hydroxy-3-oxo-4-cholestenoate | $CA^4$-7α-ol-3-one | 16 | 3515 | 516,501,*426*,269 |
| 3β,7β-Dihydroxy-5-cholestenoate | $CA^5$-3β,7β-ol[d] | 17 | 3530 | 590,*500* |
| 3β-Hydroxy-7-oxo-5-cholestenoate | $CA^5$-3β-ol-7-one[d] | 18 | 3680 | *516*,426,411,129 |

[a] C, cholestane;CA, cholestanoate; superscript indicates position of double bond; Greek letters denote configuration of hydroxyl groups.

[b] RI, Retention Index, Kovats, on a fused silica capillary column coated with cross-linked methyl silicone

[c] Intensities of charged ions with m/z values above 200-300 were enhanced relative to those of lighter fragments; base peak is shown in italics; m/z, mass/charge.

[d] Tentative identification, reference compound not available.

[e] enumerated peaks on Fig 3.

Table II shows the production of oxysterols in normal and virus transformed human fibroblasts when incubated with lipoproteins. The amounts of neutral and acidic oxygenated cholesterol derivatives were determined in the media (10 ml) containing 10% FCS (cholesterol concentration 1.2 mM) after incubation with fibroblasts for 48 hours. Cells incubated for 0.25 h served as controls.

| Table II Structure[a] | Am unt[b] of steroid found in media - *pmol* | | | |
|---|---|---|---|---|
| | Normal fibroblasts[c] | | Virus-transformed[c] fibroblasts | |
| | 0.25 h; n=2[d] | 48 h; n=4[d] | 0.25 h; n=2[d] | 48 h; n=4[d] |
| $C^5$-3β,7α-ol[e] | 171: 117-225 | 54: 39 - 81 | 156: 81 - 228 | 60: 54 - 123 |
| $C^4$-7α-ol-3-one | <6: <3 - <6 | <12: <6 - <18 | <9: <6 - <9 | 69: 51 - 159 |
| $C^5$-3β,7β-ol[e] | 138: 108 - 165 | 114: 99 - 156 | 144: 81 - 207 | 129: 75 - 216 |
| $C^5$-3β-ol-7-one[e] | 879: 840 - 915 | 816: 609-1140 | 993: 633-1350 | 867: 657-1881 |
| $C^5$-3β,24-ol | 6: <6 - 6 | 6: <6 - 9 | 3: <3 - 6 | 9: <6 - <18 |
| $C^5$-3β,25-ol[e] | 6: <3 - 12 | 45: 25 - 150 | 9: <3 - 18 | 27: 15 - 30 |
| $C^5$-3β,7α-25-ol | <3: <3 - 3 | <6: <3 - <9 | <3: <3 - 3 | <3: <3 - <3 |
| $C^4$-7α,25-ol-3-one | <6: <3 - <3 | 21: <9 - 27 | <3: <3 - <3 | <3: <3 - <3 |
| $C^5$-3β,27-ol | 12: 9 - 18 | 219: 153 - 267 | 12: 9 - 12 | 21: 15 - 33 |
| $C^5$-3β,7α,27ol | <3: <3 - <3 | <3: <3 - <3 | <3: <3 - <3 | <3: <3 - <3 |
| $C^4$-7α,27-ol-3-one | <12: <12 -<12 | 270: 144 - 321 | <9: <3 - <12 | <12: <9 - <15 |
| $C^5$-3β,7β,27ol | <3: <3 - <3 | 42: 33 - 48 | <3: <3 - <3 | <3: <3 - <3 |
| $C^5$-3β,27-ol-7-one | <9: <6 - <12 | 81: 33 - 108 | <12: <3 - <18 | <9: <6 - 15 |
| $CA^5$-3β-ol | 21: 12 - 27 | 21: 18 - 27 | 15: 12 - 15 | 15: 12 - 18 |
| $CA^5$-3β,7α-ol | 9: 6 - 9 | 6: 3 - 6 | 6: 6 - 6 | 6: 3 - 6 |
| $CA^4$-7α-ol-3-one | 21: 18 - 21 | 75: 63 - 117 | 18: 15 - 18 | 9: 6 - 15 |
| $CA^5$-3β,7β-ol | 12: 9 - 12 | 51: 45 - 72 | 3: <3 - 3 | <3: <3 - <3 |
| $CA^5$-3β-ol-7-one | <3: <3 - <3 | 27: 18 - 30 | <3: <3 - <3 | <3: <3 - <3 |

[a] For abbreviations and steroid names see Table I

[b] Expressed as median: range, < = an amount at or below detection limit

[c] Protein contents of normal and virus-transformed fibroblasts were 0.6 mg and 1.4 mg respectively. The size of the incubation dishes was 57

$cm^2$. All cells had been preincubated for 24 h in media containing 10% LDS.

[d] n = number of incubations.

[e] Can also be formed by autooxidation of cholesterol during incubations or during purification of samples.

Fig. 3 shows a GC/MS analysis of neutral $C_{27}$-steroids isolated from the medium after incubating normal fibroblasts with lipoproteins.

As can be seen from Table I, when normal fibroblasts are incubated for 28-48 hours with lipoproteins (media containing 10% FCS), 13 neutral and 5 acidic $C_{27}$ steroids were found in the media.

The identification was based on the GC retention indices of the derivatives and mass spectra, which were compared with those of the authentic steroids. Most of the steroids had additional oxygen groups both at C-7 and in the side chain. No additional steroids were identified in the cell extracts, and, with the exception of the autooxidation products of cholesterol (see above), the amounts of oxysteroids in the cell extracts were low and barely detectable (<10-20% of those in media). Because of this, oxysterols in the cells were usually not analyzed, unless otherwise stated.

The quantitative results after incubating normal and transformed fibroblasts for 48 hours with lipoproteins can be seen from Table II. In addition to 27-hydroxycholesterol which is formed from LDL cholesterol (Axelson 1995 supra), the amounts of three other 27-hydroxylated sterols also increased 10-20-fold when incubating normal fibroblasts. These sterols were 7α,27-dihydroxy-4-cholesten-3-one, 27-hydroxy-7-oxocholesterol and 7β,27-dihydroxycholesterol. In fact, the former of these was the major oxysterol formed. The amounts of corresponding $C_{27}$-acids also increased (about 5-10-fold). Also, 25-hydroxycholesterol and 7α,25-dihydroxy-4-cholesten-3-one increased, but the amounts of the former varied considerably, possibly indicating that a part of this sterol had been produced by autooxidation of cholesterol. A decrease of the amount of 7α-hydroxycholesterol

was also noted indicating a consumption of this sterol during the incubations. The amounts of the other steroids were similar to those of the controls, suggesting that they were present in FCS when added to the media.

Much smaller amounts of oxysterols were formed by the transformed fibroblasts (Table II). For example, the amounts of 27-hydroxycholesterol only increased about 2-fold, and the other 27-hydroxylated sterols were barely detectable in the media. Only the amounts of 7α-hydroxy-4-cholesten-3-one increased significantly (this was not the case in normal fibroblasts) which may be related to a decrease of the amount of its potential precursor 7α-hydroxycholesterol. These results indicated that 27-hydroxylation of sterols may be obstructed in transformed fibroblasts (see below).

The oxysterols were also studied with regard to the time course of their cellular production, as shown in Table III which shows time-response for the production of oxysterols in normal fibroblasts when incubated with lipoproteins. The amounts of neutral oxygenated cholesterol derivatives were determined in the media (15 ml) containing 10% FCS (cholesterol concentration 1.2 mM) after incubation for 0.25 h with normal human fibroblasts (protein content 1.7 mg, size of dishes 143 $cm^2$) All cells had been preincubated for 24 h in media containing 10% LDS.

TABLE III

| Structure[a] | Amount (*pmol* )of oxysterol found in media after incubation for: | | | |
|---|---|---|---|---|
| | 0.25 h | 3 h | 8 h | 24 h |
| $C^5$-3β,7α-ol[b] | 92 | 92 | 31 | 15 |
| $C^4$-7α-ol-3-one | 55 | 65 | 58 | 45 |
| $C^5$-3β,7β-ol[b] | 92 | 92 | 58 | 66 |
| $C^5$-3β-ol-7-one[b] | 508 | 500 | 369 | 412 |
| $C^5$-3β,24-ol | 15 | 15 | 10 | 10 |
| $C^5$-3β,25-ol[b] | 28 | 28 | 10 | 15 |
| $C^5$-3β,7α,25-ol | 25 | 20 | 5 | 5 |
| $C^4$-7α,25-ol-3-one | 33 | 28 | 25 | 30 |
| $C^5$-3β,27-ol | 23 | 30 | 70 | 240 |
| $C^5$-3β,7α,27-ol | 2 | 2 | 1 | 1 |
| $C^4$-7α,27-ol-3-one | 65 | 58 | 138 | 308 |
| $C^5$-3β,7β,27-ol | 3 | 3 | 15 | 13 |
| $C^5$-3β,27-ol-7-one | 8 | 5 | 43 | 108 |

[a] For abbreviations and steroid names, see Table I

[b] Can also be formed by autooxidation of cholesterol during the incubations or during purification of samples.

As shown in Table III incubation of normal human fibroblasts for different lengths of time showed that the formation of 27-hydroxylated sterols had started 3-8 h after exposure to lipoproteins. No production of 25-hydroxylated sterols was observed during the first 24 h. A decrease of the amounts of 7α-hydroxycholesterol was noted after 8 h of incubation.

Example 2

Metabolism of LDL cholesterol and 7-oxygenated sterols in normal fibroblasts

The cell lines, cell culture conditions, analysis of oxysterols and steroid acids and the HPLC procedures were as in Example 1.

The metabolism of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-oxocholesterol was studied by incubating these sterols (5 nmol) with normal human fibroblasts (protein content about 0.5 mg, dish size 57 $cm^2$) for 24 or 48 h in media (10 ml) containing 10% LDS. The values given in percent represent the distribution of observed metabolites.

When 7α-hydroxycholesterol was incubated (24 h), the major metabolites found were 7α-hydroxy-4-cholesten-3-one (57%) and 7α,27-hydroxy-4-cholesten-3-one (43%) (acids were not analyzed). Only a small amount of 7α,27-hydroxy-cholesterol (0.5%) was noted suggesting that oxidation/isomerization of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one precedes 27-hydroxylation. The corresponding enzyme activities have been found previously in fibroblasts (Skrede et al (1986) J Clin Invest 78, 729). When 7α-hydroxy-4-cholesten-3-one was incubated with the fibroblasts for 48 h, the steroid was extensively converted to 7α,27-dihydroxy-4-cholesten-3-one (37%) and 7α-hydroxy-3-oxo-4-cholestenoic acid (63%). A small amount of 25-hydroxylated 7α-hydroxy-4-cholesten-3-one (0.5%) was also found. These results show that 7α-hydroxycholesterol can be converted to 7α-hydroxy-4-cholesten-5-one, 7α,27-dihydroxy-4-cholesten-3-one, and 7α-hydroxy-3-oxo-4-cholestenoic acid by normal fibroblasts and this could explain the appearance of these metabolites and the disappearance of 7α-hydroxycholesterol in media during the incubations with FCS (Table II).

The metabolism of 7β-hydroxycholesterol differed from that of 7α-hydroxy-cholesterol. When this sterol was incubated for 48 h with normal fibroblasts, the major metabolites were 7β,27-dihydroxycholesterol (1%) and 3β,7β,-dihydroxy-5-

cholestenoic acid (62%). In addition, a large portion (about one-third) of 7β-hydroxycholesterol was converted to 7-oxocholesterol (20%), 27-hydroxy-7-oxocholesterol (1%), and 3β-hydroxy-7-oxo-5-cholestenoic acid (14%). Oxidation of 7β-hydroxy group also occurred when 7β,27-dihydroxycholesterol was incubated.

Surprisingly, no conversion of 7β-hydroxycholesterol to the corresponding 3-oxo-$\Delta^4$ derivative was observed, which was consistent with the absence of 7β-hydroxylated 3-oxo-$\Delta^4$ steroids in media after incubating fibroblasts with FCS (Table I). This implies that prior art 7β-hydroxycholesterol derivatives are unable in vivo to mimic the effects of the compounds of the invention.

Incubation of 7-oxocholesterol with normal fibroblasts resulted in the formation of 27-hydroxy-7-oxocholesterol (55%) and the corresponding $C_{27}$-acid (35%). A small amount was converted to 7β-hydroxycholesterol (10%), but a conversion to 7α-hydroxylated products was not observed. Thus, the formation of $C_{27}$-steroids having oxygen groups in both the 7- and 27-positions by normal fibroblasts could be due to the presence of autooxidation products of cholesterol in the medium. However, this did not exclude the possibility that 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenoic acid could also be derived from 27-hydroxycholesterol. 7α-Hydroxylation of 27-hydroxycholesterol in human fibroblasts was first noted by the present applicants (Axelson et al. (1995) J. Lipid Res. 36, 290) and was later found to be occurring generally in these cells (Zhang et al. (1995) Biochem. Biophys. Acta. 1256, 353). The product 7α,27-dihydroxycholesterol is extensively converted to 7α,27-dihydroxy-4-cholesten-3-one and the corresponding acid in the cells.

In order to determine whether LDL cholesterol (via 27-hydroxycholesterol) could be converted to 7α,27-dihydroxy-4-cholesten-3-one and the acid, the contribution from 7α-hydroxycholesterol (which is always present when the medium contains lipoproteins) had to be accounted for. This was made possible by the following

experiment. LDL and other lipoproteins in FCS were first labeled with [$^3$H]cholesteryl oleate and were then incubated with normal fibroblasts in the presence and absence of cyclosporin A (CsA), a selective inhibitor of the sterol 27-hydroxylase (Axelson 1995 supra, Princen et al. (1991) Biochem J. 275, 501. Dalbäck-Sjöberg et al. (1993) Biochem. J. 293, 203) When lipoproteins are labeled in this way, the cellular uptake of [$^3$H]cholesteryl oleate is due solely to an LLD receptor-dependent process (i.e. a physiological uptake of LDL (Axelson 1995 supra). After the incubations, radioactive 27-hydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one, and 7α-hydroxy-3-oxo-4-cholestenoic acid were analyzed by HPLC. $^3$H-Labeled 7α-hydroxy-4-cholesten-3-one, the direct metabolite of 7α-hydroxycholesterol, was also determined. If $^3$H-labeled 7α-hydroxycholesterol (free or esterified) was present in the medium, its 3-oxidized metabolite was expected to accumulate in the presence of CsA, since the drug prevented further metabolism (see above). The amount of metabolite would then reflect the contribution to 7α,27-dihydroxy-4-cholesten-3-one and the acid from 7α-hydroxycholesterol in the absence of CsA. Obviously, essentially no formation of 27-hydroxylated compounds was expected in the presence of CsA (see also Fig. 1). For comparison, fibroblasts were also incubated with lipoproteins labeled with [$^3$H]cholesterol, whose cellular uptake is not entirely dependent on LDL receptors.

The results of these incubations are summarized in Table IV, which shows the formation of radioactive metabolites from LDL [$^3$H ]cholesteryl oleate in normal fibroblasts. The amounts of $^3$H-labeled 27-hydroxycholesterol, 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenoic acid were determined in media and cells after incubating normal human fibroblasts (protein content, 0.7 mg; size of dishes, 57 cm$^2$) for 68 h with media (10 ml) containing LDL (4% FCS; cholesterol concentration, 1.2 mM) labeled with [$^3$H]cholesteryl oleate or [$^3$H]cholesterol. When 27-hydroxylation of sterols was obstructed by cyclosporin A (CsA, 10 μM), the accumulation of $^3$H-labeled 7α-hydroxy-4-cholesten-3-one indicated the presence of autooxidized [$^3$H]cholesterol or [$^3$H]cholesteryl oleate (i.e. free or esterified 7α-hydroxycholesterol) in the media during the incubation.

TABLE IV

| Structure[a] | Amount of $^3$H -labeled oxysterol found[b] - *dpm* | | | |
|---|---|---|---|---|
| | FCS + [$^3$H]cholesteryl oleate[c] | | FCS + [$^3$H]cholesterol[c] | |
| | Control | +CsA | Control | +CsA |
| $C^5$-3β,27-ol | 12 000 | <1 970 | 7 090 | <120 |
| $C^4$-7α,27-ol-3-one | 5 140 | <1 140 | 88 800 | 12 800 |
| $CA^4$-7α-ol-3-one | 10 800 | <120 | 26 300 | 1 690 |
| $C^4$-7α-ol-3-one | <410 | <410 | 1 450 | 25 300 |

[a] For abbreviations and steroid names, see Table I,

[b] Values represent the sum of the amounts found in medium and cells; < = an amount at or below detection level

[c] FCS was preincubated for 16 h at 20°C with [$^3$H]cholesteryl oleate (48 x $10^6$ dpm) or [$^3$H]cholesterol (49 x $10^6$ dpm). All cells had been preincubated for 24 h in media containing 10% LDS.

In addition to 27-hydroxycholesterol, both 7α,27-dihydroxy-4-cholesten-3-one and 7α-3-oxo-4-cholestenoic acid were found as $^3$H-labeled compounds after incubation with [$^3$H]cholesteryl oeate. The HPLC analyses of these metabolites are shown (Fig. 4). Since no acccumulation of radioactive 7α-hydroxy-4-cholesten-3-one was observed in the corresponding incubation with CsA (Table IV), autooxidation (7α-hydroxylation) of [$^3$H]cholesteryl oleate had not occurred during the incubations. In the incubations with [$^3$H]cholesterol, much larger amounts of radiactive 7α,27-dihydroxy-4-cholesten-3-one and the corresponding acid were found, although the amount of 27-hydroxycholesterol was less than with [$^3$H]cholesteryl oleate. However, the incubation with [$^3$H]cholesterol and CsA resulted in a significant accumulation of $^3$H-labeled 7α-hydroxy-4-cholesten-3-one suggesting that most of the 27-oxygenated metabolites had been produced from autooxidized [$^3$H]cholesterol (7α-hydroxycholesterol). The difference in chemical stability toward oxygen between [$^3$H]cholesteryl oleate and [$^3$H]cholesterol was surprising, but was

confirmed by exposing them to air and heat in an aqueous/methanolic environment for 24 h. No autooxidation products (<0.1%) of [$^3$H]cholesteryl oleate could be detected, whereas about 2% of [$^3$H]cholesterol were autooxidized. Thus, the results demonstrate that LDL cholesteryl esters are hydrolyzed and are converted to 27-hydroxycholesterol, which is then 7α-hydroxylated and oxidized to 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenoic acid in normal human fibroblasts. The latter seems to be the major metabolic end product of this extended LDL pathway in fibroblasts.

Example 3:

Metabolism of LDL cholesterol and side-chain hydroxylated sterols in transformed fibroblasts

The cell lines, cell culture conditions, analysis of oxysterols and steroid acids and the HPLC procedures were as in Example 1.

In contrast to normal fibroblasts, only small amounts of 27-oxygenated sterols were detected in media after incubating transformed human fibroblasts with lipoproteins (Table II). Although the increased amounts of 7α-hydroxy-4-cholesten-3-one could indicate that 27-hydroxylation of sterols was obstructed in these cells (see above), the lack of 27-hydroxylated sterols in the media could also be due to a decreased cellular uptake of LDL and oxysterols, or to an increased formation of conjugates (e.g. fatty acid esters or sulfate esters).

Table V shows the distribution of $^3$H-labeled cholesterol and cholesteryl esters after incubating normal and transformed fibroblasts with lipoproteins labeled with radioactive cholesterol or cholesteryl oleate for 48 h. Distribution of radioactivity after incubating normal and virus-transformed human fibroblasts for 48 hours in media containing lipoproteins (10% FCS) labeled with [$^3$H]cholesteryl or [$^3$H]cholesteryl oleate. The total concentration of unlabelled cholesterol in FCS was 1.2 mM. Incubations for 0.25 h served as controls.

TABLE V

| Incubation conditions[a] | | Distribution of free & esterified [$^{3}$H] cholesterol[c] | | | | | |
|---|---|---|---|---|---|---|---|
| Time | Addn[b] | In the Medium | | | In the Cells | | |
| *h* | | Free | Esters | Total | Free | Esters | Total |
| | | normal cells | | | | | |
| 0.25 | C/FCS[d] | 95 | 4 | 99 | 1 | <1 | 1 |
| 48 | C/FCS[d] | 82 | 3 | 85 | 11 | 4 | 15 |
| 48 | C/FCS[d] | 80 | 4 | 84 | 11 | 4 | 16 |
| 0.25 | CO/FCS[d] | 2 | 98 | 100 | <1 | <1 | <1 |
| 48 | CO/FCS[d] | 10 | 81 | 91 | 6 | 4 | 9 |
| 48 | CO/FCS[d] | 11 | 77 | 88 | 9 | 3 | 12 |
| | | transformed cells | | | | | |
| 0.25 | C/FCS[d] | 95 | 4 | 99 | 1 | <1 | 1 |
| 48 | C/FCS[d] | 66 | 12 | 79 | 17 | 4 | 21 |
| 48 | C/FCS[d] | 73 | 5 | 78 | 18 | 4 | 22 |
| 0.25 | CO/FCS[d] | 1 | 99 | 100 | <1 | <1 | <1 |
| 48 | CO/FCS[d] | 38 | 49 | 86 | 11 | 3 | 14 |
| 48 | CO/FCS[d] | 34 | 51 | 84 | 12 | 3 | 16 |

[a] Protein content of normal and virus transformed fibroblasts were 0.6 mg and 1.4 mg respectively. The size of the incubation dishes was 57 cm$^2$. All cells had been preincubated for 24 h in media containing 10% LDS. Results from these incubations are also shown in Table II.

[b] Additions to the incubation medium : The amounts of [$^{3}$H] cholesterol and [$^{3}$H] cholesteryl oleate added to the medium were 22.8 x $10^6$ dpm and 18.9 x $10^6$ dpm respectively. The sterols were preincubated with FCS for 16 h at 20°C.

[c] Percentage of recovered radioactivity. The total recovery was >90% of the radioactivity added.

[d] C/FCS: [$^{3}$H]Cholesterol in FCS, CO/FCS: [$^{3}$H]Cholesterol oleate in FCS

Results on the oxysterol production from the same incubations are those shown in Table II. As seen in Table V, the cellular uptake and retention (cell content) of [$^3$H]cholesterol in normal and transformed cells were about 16 and 22%, respectively. About 4% had been esterified by both cell types. After the incubations with [$^3$H]cholesteryl oleate, the retention of the compound was about 11% in the normal cells and 15% in the transformed cells, although a major portion had been hydrolyzed in the cells. About 9% and 35% of hydrolyzed [$^3$H]cholesterol were also present in media of the normal and transformed fibroblasts, respectively, due to an efflux of hydrolyzed LDL cholesterol from the cells (Axelson 1995 supra, Fielding et al 1995 J Lipid Res 36, 211-228). When the cellular content and the efflux of cholesterol in the incubations with [$^3$H]cholesteryl oleate in the normal and the transformed cells was 19% (32%/mg of protein) and 50% (36%/mg of protein), respectively. These results show that the uptake of LDL (reflected by that of [$^3$H]cholesteryl oleate) was not decreased but possibly increased in the transformed cells also when the protein content of the cells was taken into account. Thus, a possible reduced formation of 27-hydroxylated sterols by transformed fibroblasts (Table II) was not due to a decreased uptake of LDL.

In order to determine whether the shortage of 27-hydroxy-cholesterol and other 27-hydroxylated sterols in media of transformed fibroblasts could be due to an increased metabolism (other than formation of $C_{27}$-acids) or conjugation, the metabolism of 25-hydroxycholesterol was studied. The major reason for selecting this sterol instead of 27-hydroxycholesterol was that 25 hydroxycholesterol was available in a $^3$H-labeled form, so that major metabolites or conjugates could be traced and would not escape detection. Because of their similarities in structure (both having a 3β-hydroxy-$\Delta^5$ structure and one hydroxyl group in the side chain), the cellular handling of the two sterols was expected to be similar (except that the 25-hydroxy group could not be oxidized to a carboxyl group). Table VI shows the metabolism of 25-[$^3$H]hydroxycholesterol in normal and transformed fibroblasts. In particular it shows the distribution of recovered radioactivity after incubating normal and transformed fibroblasts for 48 h with media containing $^3$H-labeled and

unlabeled 25-hydroxycholesterol and 10% LDS (cholesterol concentration 0.1 mM) Incubations for 0.25 h served as controls.

TABLE VI

| Radioactive comp. | Relative amount found[a b] | | | | | |
|---|---|---|---|---|---|---|
| | Normal fibroblasts | | | Transformed fibroblasts | | |
| | 0.25 h | 48 h | 48 h | 0.25 h | 48 h | 48 h |
| In the medium | | | | | | |
| 25-[$^3$H] hydroxycholesterol | 47 | 5 | 4 | 73 | 21 | 21 |
| Non-polar metabolites[c] | <1 | <1 | <1 | <1 | 2 | 2 |
| Polar metabolites[d] | 2 | 43 | 37 | 2 | 2 | 2 |
| Total radioactivity[e] | 57 | 57 | 51 | 79 | 30 | 29 |
| In cells | | | | | | |
| 25-[$^3$H] hydroxycholesterol | 35 | 30 | 34 | 18 | 49 | 50 |
| Non-polar metabolites[c] | <1 | <1 | <1 | <1 | 5 | 5 |
| Polar metabolites[d] | <1 | 4 | 5 | <1 | 2 | 3 |
| Total radioactivity[e] | 43 | 43 | 49 | 22 | 70 | 71 |

[a] The amounts of $^3$H-labeled and unlabeled 25-hydroxycholesterol added to the medium (10 ml containing 10%LDS) were 1.4 x $10^6$ dpm and 1.2 nmol respectively. Protein contents of normal and virus transformed fibroblasts were 0.6 mg and 1.4 mg respectively. The size of the incubation dishes was 57 cm$^2$. All cells had been preincubated for 24 hours in media containing 10%LDS.

[b] Figures represent % recovered radioactivity. The total recovery was about 90% of the radioactivity added.

[c] Retention time on straight phase HPLC was 3.5 - 4.0 min using hexane/isopropyl alcohol (97:3) as the mobile phase and a flow rate of 1 ml x min$^{-1}$. It was tentatively identified as 25-hydroxycholesterol esterified with a fatty acid.

[d] retention time on reversed phase HPLC was 3.0 - 4.5 min using methanol/ethanol/water (80:20:10) as the mobile phase and a flow rate of 1 ml x min $^{-1}$. In this fraction, 7α,25-dihydroxy-4-cholesten-3-one was identified by gas chromatography-mass spectrometry, and the amounts were similar to those calculated from the radioactivity.

[e] Also includes other radioactive compounds, each accounting for less than 1% of the total recovery.

In addition to 25-[$^3$H]hydroxycholesterol, two major radioactive metabolites, one polar and one nonpolar, were found by HPLC. Other metabolites constituted less than 1% each of the recovered radioactivity. No radioactivity (<0.1%) corresponding to oxidized 25-[$^3$H]hydroxycholesterol without a 7α-hydroxy group (i.e. 25-hydroxy-4-cholesten-3-one, see below) was found. Neither did we find any radioactivity (<1%) in fractions containing weak acids (e.g. having a free carboxyl group) or stronger acids (e.g. glucuronides or mono- or disulfates) which were isolated from the extracts by anion exchange chromatography. After collecting a fraction of the HPLC effluent containing the polar metabolite and derivatization, it was identified by GC/MS as 7α,25-dihydroxy-4-cholesten-3-one (Zhang supra). The nonpolar metabolite(s) had a retention time (3.7 min), which was similar to that of the 3-acetate derivative of 25-hydroxycholesterol (retention time 4.9 min) by straight-phase HPLC (retention time of free 25-hydroxycholesterol was 11.8 min). It was therefore tentatively characterized as being fatty acid esters of 25-$^3$H]hydroxycholesterol. This was supported further by recovery of free 25-[$^3$H]hydroxycholesterol after treating the nonpolar metabolite(s) with mild alkali in a methanolic solution. Table VI clearly reveals large differences in the handling of 25-[$^3$H]hydroxycholesterol between the two cell lines. Intact 25-[$^3$H]hydroxycholesterol was found mainly in the cells (32% in the normal and 50% in the transformed cells). A large portion (about 43%; 71%/mg of protein) of 25-[$^3$H]hydroxycholesterol had been converted to 7α,25-dihydroxy-4-cholesten-3-one by normal cells (62) but much less so (about 3%; 2%/mg of protein) by the transformed cells. This sterol was recovered mainly in the media. On the other hand, esterification of 25-[$^3$H]hydroxycholesterol was noted only in the transformed cells,

although the amount of esters was relatively small (about 7%). These results show that 25-hydroxycholesterol is readily taken up by both normal and transformed cells, but whereas the sterol is extensively 7α-hydroxylated in normal cells, this reaction is obstructed in transformed cells. The results also suggested that the apparent shortage of 27-hydroxycholesterol in transformed cells was due to a decreased formation rather than an increased conjugation, since only a minor amount of the analogous sterol 25-hydroxycholesterol was esterified and since no other conjugates were found.

After these observations, the rates of oxidation/isomerization of 3β,7α-dihydroxy-$\Delta^5$ steroids to 3-oxo-$\Delta^4$ steroids in normal and transformed cells were also investigated. 7α,27-Dihydroxycholesterol (1.2 nmol) was therefore incubated with normal and transformed fibroblasts (protein contents 0.4 and 1.1 mg, respectively, size of dishes 57 $cm^2$) for 48 h in media (10 ml) containing 10% LDS, and the metabolites were then analyzed by GC/MS. Incubations for 15 min served as controls. The analyses showed that this sterol was readily taken up by the cells, since only about 1% remained in the media. In media from normal cells, about 26% and 39% were recovered as 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-3-oxo-4-cholestenoic acid, respectively. The corresponding values for the transformed cells were 28% and 19%. Trace amounts (about 1%) were converted to 3β,7α-dihydroxy-5-cholestenoic acid in the transformed cells. No 7-oxo-, 7β-hydroxy-, or other metabolites were found. Thus, almost the same amount of 7α,27-dihydroxycholesterol were oxidized by the normal and transformed fibroblasts. However, when the number of incubated cells (cell protein content) were taken into account, the oxidation rate in transformed cells was calculated to be about 25% of that of normal cells. These studies show that the apparent activities of 27- and 7α-hydroxylating enzymes are much lower in transformed than in normal fibroblasts (estimated to be <2% of the normal activity when corrected for the cellular protein content or total uptake of LDL), whereas the enzyme catalyzing oxidation of 3β-hydroxy-$\Delta^5$ sterols is affected to a lesser extent.

Example 4:

Effects of LDL and oxysterols on HMG-CoA reductase in normal and tumor-transformed human fibroblasts

Normal human fibroblasts (line GM 08333) from NiGMS, Corriell Institute for Medical Research (Camden NJ) and SV-40 virus transformed human fibroblasts (90-VA VI), a kind gift from Dr. Stein (University of Colorado, Boulder, CO), were grown in monolayers in tissue culture flasks maintained in a 95% air/5% $CO_2$ atmosphere at 37°C in a humidified incubator. Cells were cultured in Dulbecco´s Minimal Eagle´s Medium supplemented with essential and non-essential amino acids and 10% fetal calf serum (FCS, from Life Technologies, Inc., Stockholm, Sweden). Cells were then seeded in dishes (20 $cm^2$) at a density of 5,000 cells per $cm^2$ and the media (5 ml) contained 10% FCS. The experiments were started 48-72 h later, at which time a cell density of approximately 20,000 per $cm^2$ had been reached (the cells were subconfluent also at the end of the incubations). All cells were then incubated for 24 h in medium (5 ml) containing 10% lipoprotein deficient serum, prepared by treating FCS with Cab-O-Sil (Weinstein (1979) Circulation 54, Suppl. II 59), before LDL (0.5-8% FCS; cholesterol concentration, 1.2 mM) and oxysterols (tested at a concentration of 0.12 μM) were added to the incubation media, the latter in freshly prepared ethanol solutions. The ethanol concentrations of the media then became 0.2%. Control cells were incubated in the same way but without sterols. After an incubation period of 24 h, the cells were rinsed twice with phosphate buffered saline and harvested for assay of cellular HMG-CoA reductase activity as described in Cavance et al 1981 J Biol Chem 256, 2675. In brief, cell lysate were incubated in 200 mM potassium phosphate, 20 mM dithiotheritol, 40 mM glucose-6-phosphate, 5 mM NADPH, and 5 units/ml of glucose-6-phosphate dehydrogenase. After a 15-min preincubation at 37°C, 0.9 nmol/L [14C]HMG-CoA (57mCi/mmol) and unlabelled HMG-CoA (the final concentration of HMG-CoA was 100 μM) were added for a 60-min incubation at 37°C. The final reaction volume for each sample was 60 μL. The reaction was stopped by addition of 5 μL 5 M HCl which also allowed lactonization of the produced [14C]mevalonate. After addition of known amounts of [3H]m valonolactone (internal standard) one aliquot

was used to separate [14C]mevalonolactone from [14C]HMG-CoA by ion-exchange chromatography (Edwards et al 1977 J. Lipid Res. 20, 40) and one aliquot was used for spectrophotometric determination of protein content. The [3H]- and [14C]-radioactivity was analyzed by a scintillation counter which was equipped with a program for automatic correction for quenching and spill-over (Beckman LS 5000TA).

Table VII shows the effects of LDL and oxysterols on HMG-CoA reductase in normal and transformed fibroblasts. In particular it shows the effects of LDL (0.5-8% FCS; cholesterol concentration 1.2 mM) and of oxysterols (0.12 $\mu$M in media containing 10% LDS) on HMG-CoA reductase in normal and virus transformed human fibroblasts.. The activities of HMG-CoA reductase were determined after incubating the fibroblasts for 24 h. All cells were preincubated for 24 h in media containing 10% LDS.

TABLE VII

| Structure[a] | Observed metab lism[b] | HMGCoA reductase activity[c] | | ΔSupp-ression[d] % |
|---|---|---|---|---|
| | | Normal cells | Trans-formed | |
| LDL cholesterol | 27-hydroxylation | 15-31 | 40-88 | 32-57 |
| $C^4$-7$\alpha$-ol-3one | 27-hydroxylation | 26 | 50 | 24 |
| $C^5$-3$\beta$,7$\beta$-ol | 7-oxidation or 27-hydroxylation | 39 | 60 | 21 |
| $C^5$-3$\beta$-ol-7-one | 27-hydroxylation | 38 | 100 | 62 |
| 5$\alpha$-$C^{8(14)}$-3$\beta$-ol-15-one | 27-hydroxylation | 22 | 41 | 19 |
| $C^5$-3$\beta$,25-ol | 7$\alpha$-hydroxylation | 37 | 77 | 40 |
| $C^5$-3$\beta$,27-ol | 7$\alpha$-hydroxylation | 33 | 79 | 46 |
| $C^5$-3$\beta$,7$\alpha$,25-ol | 3-oxidation/ isomerizn | 23 | 49 | 26 |
| $C^5$-3$\beta$,7$\alpha$,27-ol | 3-oxidation/ isomerizn | 30 | 60 | 30 |
| $C^4$-27-ol-3-one | 27-oxidation to acid | 31 | 38 | 7 |
| $C^4$-7$\alpha$,27-ol-3-one | 27-oxidation to acid | 33 | 34 | 1 |
| $C^5$-3$\beta$,27-ol-7-one | 27-oxidation to acid | 7 | 33 | 26 |
| $C^4$-25-ol-3-one | none | 33 | 37 | 4 |
| $C^4$-7$\alpha$,25-ol-3-one | none | 26 | 31 | 5 |
| $CA^4$-7$\alpha$-ol-3-one | none | 30 | 47 | 17 |

[a] For abbreviations and steroid names see Table I

[b] Metabolism observed in normal human fibroblasts. The activities of the enzymes catalysing 27-hydroxylation, 7α-hydroxylation and 3-oxidation with isomerisation of the 5-double bond of sterols were shown to be reduced in tumour-transformed fibroblasts.

[c] % of control. The activities of HMG-CoA reductase in normal and transformed fibroblasts were 58 and 96 pmol/min/mg protein respectively.

[d] Difference in degree of suppression of HMG-CoA reductase in normla and transformed fibroblasts induced by LDL or the oxysterol

By relating the intracellular production of different oxysterols to the observed suppression of HMG-CoA reductase in normal and transformed fibroblasts and using inhibitors of sterol-metabolizing enzymes, it was apparent that:

1. LDL cholesterol and a number of oxysterols (including) 27-hydroxycholesterols, 3β-hydroxy-5α-cholest-8(14)-en-15-one, and the autooxidation products of cholesterol 25-hydroxycholesterol, 7-oxo-cholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol), which have been considered to be potent suppressors of HMG-CoA reductase, all appear to have to be metabolized prior to being biologically active.

2. In contrast, their metabolites 7α,27-dihydroxy-4-cholesten-3-one, 3β,27-dihydroxy-5-cholesten-7-one and 7α,25-dihydroxy-4-cholesten-3-one (and most likely 3β,27-dihydroxy-5α-cholest-8(14)-en-15-one) do not seem to require further metabolism in order to be active, i.e. are true suppressors of HMG-CoA reductase.

3. Of the above compounds, one was remarkably more potent in the inhibition of HMG-CoA reductase and thus an additional aspect of the invention provides the compound 3β,27-dihydroxy-5-cholesten-7-one, preferably in substantially pure form, for instance >75%, preferably > 90% pure and most preferably >95% enantiomerically pure. This aspect of the invention further provides the use in medicine of this compound and/or its active metabolites such as the cholestenoate. A preferred use is in the manufacture of medicament for suppressing HMG-CoA reductase activity in a cell or mammal, including humans. Representative for this aspect of the invention include its administration to a human or mammal to reduce serum cholesterol and/or the biosynthesis of cholesterol.

4. Certain sterols with these structures also showed suppression of HMG-CoA reductase in other human neoplastic cells, including breast carcinoma, colonic carcinoma and malignant melanoma cells, which all displayed a defective regulatory response to LDL. This is shown in Example 5.

Example 5:

Effects of LDL and oxysterols on HMG-CoA reductase in human malignant cells

Human breast carcinoma (MDA 231), colonic carcinoma (WiDr) and malignant melanoma (SK-MEL-2) cell lines from American Type Culture Collection, U.S.A., were grown in monolayers in tissue culture flasks maintained in a 95% air/5% $CO_2$ atmosphere at 37°C in a humidified incubator and were cultured in either Dulbecco's Modified Eagle's Medium (MDA 231) or Minimal Eagle's Medium (the other cells) supplemented with essential and non-essential amino acids and 10% fetal calf serum (FCS, from Life Technologies, Inc., Stockholm, Sweden). Cells were then seeded in dishes (20 $cm^2$) at a density of 5,000 cells per $cm^2$ and the media (5 ml) contained 10% FCS. The experiments were started 48-72 h later, at which time a cell density of approximately 20,000 per $cm^2$ had been reached (the cells were subconfluent also at the end of the incubations). All cells were then incubated for 24 h in media (5 ml) containing 10% lipoprotein deficient serum, prepared by treating FCS with Cab-O-Sil (Weinstein supra), before LDL (2% FCS; cholesterol concentration, 1.2 mM) and oxysterols (tested at a concentration of 0.12 μM) were added to the incubation media, the latter in freshly prepared ethanol solutions. The ethanol concentrations of the media then became 0.2%. Control cells were incubated in the same way but without sterols. After an incubation period of 24 h, the cells were rinsed twice with phosphate buffered saline and harvested for assay of cellular HMG-CoA reductase activity as described (Cavenel supra). In brief, cell lysates were incubated in 200 mM potassium phosphate, 20 mM dithiotheritol, 40 mM glucose-6-phosphate, 5 mM NADPH, and 5 units/mL of glucose-6-phosphate dehydrogenase. After a 15-min preincubation at 37°C, 0.9 nmol/L [14C]HMG-CoA (57 mCi/mmol) and unlabelled HMG-CoA (the final concentration of HMG-CoA was 100 μM) were added for a 60-min incubation at 37°C. The final reaction

volume for each sample was 60 μL. The reaction was stopped by addition of 5 μL 5 M HCl which also allowed lactonization of the produced [14C]mevalonate. After addition of known amounts of [3H]mevalonolactone (internal standard) one aliquot was used to separate [14C]mevalonolactone from [14C]HMG-CoA by ion-exchange chromatography (Edwards supra) and one aliquot was used for spectrophotometric determination of protein content. The [3H]- and [14C]-radioactivity was analyzed by a scintillation counter which was equipped with a program for automatic correction for quenching and spill-over (Beckman LS 5000TA).

Table VIII shows the effects of LDL and oxysterols on HMG-CoA reductase in human malignant cells. In particular it shows the effects of LDL (2% FCS; cholesterol concentration 1.2 mM and of selected oxysterols (0.12 μM in media containing 10% LDS) on HMG-CoA reductase in breast and colonic carcinoma cells and malignant melanoma cells after incubation for 24 hours. All cells had been pre-incubated for 24 h in media containing 10% LDS. For comparison the corresponding values on transformed human fibroblasts are also shown.

| TABLE VIII<br>Structure[a] | Activity of HMG-CoA reductase<br>Breast carcinoma cells | Colonic carcinoma cells | Malignant melanoma cells | Trans-formed[b] fibroblasts | Median |
|---|---|---|---|---|---|
| 3β-hydroxy-Δ$^5$ sterols | | % of control[c] | | | |
| C$^5$-3β-ol (LDL cholesterol) | 67 | 79 | 121 | 68 | 74 |
| C$^5$-3β-ol-7-one | 96 | 91 | 123 | 100 | 98 |
| 5αC$^{8(14)}$3-β-ol-15-one | 66 | 80 | 101 | 41 | 73 |
| C$^5$-3β,25-ol | -[d] | 94 | -[d] | 77 | 86 |
| C$^5$-3β,27-ol | 75 | 86 | 75 | 79 | 77 |
| C$^5$-3β,7α,27-ol | -[d] | 74 | 73 | 60 | 73 |
| 3-oxo-Δ$^4$ sterols | | | | | |
| C$^4$-25-ol-3-one | 43 | 55 | -[d] | 37 | 43 |
| C$^4$-25-ol-3-one | 48 | 54 | 59 | 38 | 51 |

[a] C, cholestane; superscript indicates postion of double bond; Greek letters denote configuration of hydroxyl groups.

[b] For comparison, the values of tumor transformed human fibroblasts are also shown (see Example 4). The corresponding values for normal fibroblasts were 18-38%.

[c] The activities of HMG-CoA reductase in breast carcinoma cells, colonic carcinoma cells, malignant melanoma cells and transformed fibroblasts were 59, 120, 137 and 86 pmol/min per mg protein respectively.

[d] not studied.

The results show that the compounds of this type are potent suppressors of cholesterol production in many different cells, including those having low activities of sterol-metabolizing enzymes. This is in contrast to most other oxysterols that have been used as HMG-CoA reductase suppressors. Like tumor cells, normal cells

such as macrophages, apparently lack the 7α-hydroxylating enzyme, which is required for the formation of HMG-CoA reductase suppressors from cholesterol or side-chain hydroxylated 3β-hydroxy-5 sterols. These cells are believed to play an important role in the development of atherosclerotic plaques when located in the blood vessel wall Although the mechanisms behind the development of atherosclerosis are not known in detail, excessive accumulation of esterified cholesterol in blood vessel wall cells is believed to be of major importance. In relation to this it should be pointed out that we have not found any evidence that this group of sterols stimulates esterification of cholesterol in cells, a reaction which seems to be triggered by side-chain hydroxylated 3β-hydroxy-Δ5 sterols (e.g. 25-hydroxycholesterol). Furthermore, in contrast to the conventional pharmacological treatment of hypercholesterolemia and atherosclerosis using competitive inhibitors of HMG-CoA reductase (compactin or lovastatin (mevinolin)), the described group of sterols also reduces the cellular uptake of LDL-cholesterol by suppressing the number of LDL receptors on the cell surface. This is shown in Example 6.

Example 6:

Effects of oxysterols on LDL-receptors of normal human fibroblasts

Normal human fibroblasts (line GM 08333) from NiGMS, Corriell Institute for Medical Research (Camden, NJ), were grown in monolayers in tissue culture flasks maintained in 95% air/5% $CO^2$ atmosphere at 37°C in an humidified incubator. Cells were cultured in Dulbecco's Minimal Eagle's Medium supplemented with essential and non-essential amino acids and 10% fetal calf serum (FCS, from Life Technologies, Inc., Stockholm, Sweden). Cells were then seeded at a density of 6,000 cells per $cm^2$ in 50 $cm^2$ dishes in the same medium (5 ml). The experiment was started 96 h later, at which time a cell density of approximately 20,000 per $cm^2$ had been reached (the cells were subconfluent also at the end of the incubations). The effect of the sterols on LDL-receptor activity (rate of high-affinity = receptor mediated degradation of LDL) of cells was then tested. Thc cells were washed twice in phosphate buffered saline and were incubated with 5 ml of medium containing 10% lipoprotein deficient serum (LDS) prepared by treating FCS with Cab-O-Sil

(Weinstein supra). (Cell growth was not negatively affected when this serum was used). The sterols 24-hydroxy-4-cholesten-3-one, 25-hydroxy-4-cholesten-3-one and 27-hydroxy-4-cholesten-3-one were then added to the incubation media (concentration 1.1 μM) in freshly prepared ethanol solutions and the ethanol concentrations of the media were then 0.2%. To the control cells were added the same volume of ethanol but without the sterols. After incubation for 19 h, 50 μg of 125I-labeled LDL (specific activity 150-380 cpm/ng of protein, prepared from $Na^{125}I$ (from Amersham, specific activity >350 Ci/mol) as described in Langer et al 1972 J Clin Invest 51, 1528 and containing less than 1% of radioactivity as free iodide) was added to the medium and the incubations continued for additional 4 h. Cellular degradation of $^{125}I$-labeled LDL was then determined from the formation of acid-soluble radioactivity in the incubation medium (Vitols et al 1984 Blood 63 1186). All incubations were carried out in duplicate. The degradation of 125I-labeled LDL in the control cells were found to be 105-109 ng/h x mg cell protein. The corresponding values for cells treated with 24-hydroxy-4-cholesten-3-one, 25-hydroxy-4-cholesten-3-one and 27-hydroxy-4-cholesten-3-one were 40-41, 58-59 and 62-69 ng/h x mg cell protein, respectively. This shows that the sterols reduced the LDL-receptor activity of the cells by approximately 50% under the conditions used.

Since it is known that cell growth is dependent on the production of mevalonate, the effects of various oxysterols, including the "new" group of potent HMG-CoA reductase suppressors, on proliferation and viability of normal and tumor-transformed human fibroblasts were also tested. In addition to the sterols described in previous examples, several sterols with a 7β-hydroxy group were included in the tests. Although these were potential metabolites of cholesterol, many of them were not produced in detectable amounts in fibroblasts (e.g. sterols with 7β-hydroxy-3-oxo-$\Delta^4$ structure). However, we have previously shown that 7β-hydroxysterols can be formed from the corresponding 7α-hydroxy sterols when incubated with isolated human liver mi[illegible]chondria (Shoda (1993) Hepatology 17, 395).

The effects of oxysterols on growth and viability of human fibroblasts are shown in Example 7. The concentrations of oxysterols used were intentionally much lower (1/5 - 1/10) than used in most other studies on cell growth inhibition or toxicity of oxysterols (Smith et al (1989) Free Radical Biology & Medicine, 7 285). This was because we did not want to cause non-specific damages to cell membranes (Sevanian (1986) Fd. Chem. Toxic. 24, 1103) (the oxysterols can "replace" cholesterol in the membranes) and thereby induce cell death. In fact, the concentration of 1.25 µM used was similar to that required for suppression of LDL-receptors on the cells (see Example 3).

Example 7:

Effects of oxysterols on proliferation and viability of normal and tumor transformed human fibroblasts

Normal human fibroblasts (line GM 08333) and SV-40 virus transformed human fibroblasts (90-VA VI) were grown in monolayers in tissue cultered flasks maintained in a 95% air/5% $CO_2$ atmosphere at 37°C in a humidified incubator and were cultured in Dulbecco's Minimal Eagle's Medium supplemented with essential and non-essential amino acids and 10% fetal calf serum (FCS). The experiments were started 24-48 h after seeding, at which time a cell density of approximately 10,000 per $cm^2$ had been reached (the cells were subconfluent also at the end of the incubations). All cells were then incubated for 24 h in the media (2 ml) lacking serum, before oxysterols (tested at concentrations 1.25 µM and 2.5 µM) were added to the incubation media in freshly prepared ethanol solutions. The ethanol concentrations of the media then became 0.5-1.0%. Control cells vere incubated with the same volumes of ethanol but without sterols. The proliferation of cells was registered by counting in a light microscope the cell number in marked areas in the dishes after incubations for 48 h (sterol concentration 2.5 µM) or 72 h (sterol concentration 1.25 µM) and cell death was recognized microscopically as detachment or lysis of cells in the monolayer cultures.

| TABLE IX Structure[a] | Cell viability (%[d]) | | | |
|---|---|---|---|---|
| | Normal cells | | Transformed cells | |
| | 1.25 μM[b] 72 h [c] | 2.5 μM[b] 48 h [c] | 1.25 μM[b] 72 h [c] | 2.5 μM[b] 48 h [c] |
| Control | 127 | 123 | 160 | 150 |
| <u>3β-hydroxy-Δ[5] sterols</u> | | | | |
| C[5]-3β,25-ol | 91 | 117 | 45 | 179 |
| C[5]-3β,27-ol | 97 | 117 | 83 | 144 |
| <u>3β-7α-dihydroxy-Δ[5] sterols</u> | | | | |
| C[5]-3β,7α-ol | 60 | 140 | 40 | 65 |
| C[5]-3β,7α,25-ol | 119 | 117 | 43 | 62 |
| C[5]-3β,7α,27-ol[e] | 94 | 105 | 43 | 27 |
| <u>3β,7β-dihydroxy-Δ[5] sterols</u> | | | | |
| C[5]-3β,7β-ol[e] | 156 | 77 | 144 | 131 |
| C[5]-3β,7β,25-ol[e] | 140 | 62 | 34 | 36 |
| C[5]-3β,7β,27-ol | 121 | 138 | 130 | 137 |
| <u>3β-hydroxy-7-oxo-Δ[5]sterols</u> | | | | |
| C[5]-3β-ol-7-one | 163 | 128 | 118 | 209 |
| C[5]-3β,27-ol-7-one | 112 | 117 | 64 | 102 |
| <u>3-oxo-Δ[4] sterols</u> | | | | |
| C[4]-25-ol-3-one | 85 | 127 | 46 | 162 |
| C[4]-27-ol-3-one | 91 | 117 | 58 | 135 |
| <u>7α-hydroxy-3-oxo-Δ[4]sterols</u> | | | | |
| C[4]-7α-ol-3-one | 127 | 92 | 78 | 45 |
| C[4]-7α,25-ol-3-one | 119 | 76 | 6 | 0 |
| C[4]-7α,27-ol-3-one[e] | 108 | 87 | 83 | 93 |
| <u>7β-hydroxy-3-oxo-Δ[4]sterols</u> | | | | |
| C[4]-7β-ol-3-one | 164 | 89 | 90 | 74 |
| C[4]-7β,25-ol-3-one | 121 | 97 | 10 | 0 |
| C[4]-7β,27-ol-3-one | 122 | 62 | 11 | 1 |

[a] C, cholestane; superscript indicates position of double bond; Greek letters denote configuration of hydroxyl groups.

[b] Concentration of sterol in medium

[c] Incubation period

[d] Number of viable cells in relation to the number of cells at the beginning of the experiment (in %)

[e] Inconsistent effects of sterol on transformed fibroblasts (noted in other experiments)

It is apparent from the above that:

1. Tumor-transformed fibroblasts were selectively affected by the oxysterols when compared with normal cells, resulting in not only ceased growth but also in cell death of the former cells. The effect on the normal cells was relatively small.

2. 7-hydroxylated sterols are more potent than sterols lacking a hydroxyl group or having an oxo group in this position. Surprisingly, the latter group included the potent HMG-CoA reductase suppressors 25-hydroxy-4-cholesten-3-one, 27-hydroxy-4-cholesten-3-one and 3β,27-dihydroxy-5-cholesten-7-one, suggesting that the induction of cell death was not mainly due to a suppression of HMG-CoA reductase.

3. Sterols with an hydroxy group in the side chain were more potent than the corresponding sterols without such a group.

4. Highly potent inducers of death in the transformed cells were 7-hydroxy-$\Delta^4$-sterols with a hydroxyl group in the side chain (i.e. 7α,25-dihydroxy-4-cholesten-3-one, 7β,25-dihydroxy-4-cholesten-3-one and 7β,27-dihydroxy-4-cholesten-3-one). One of the sterols belonging to this group, 7α,27-dihydroxy-4-cholesten-3-one, was less potent in this assay than the others. Possibly, this was due

to a more rapid metabolism (it was converted into the corresponding C27-acid) by the transformed fibroblasts.

5. The strong lethal effect of 7-hydroxy-$\Delta^4$-sterols with a hydroxyl group in the side chain was not limited to tumor-transformed fibroblasts, but was also seen in other human malignant cells, as shown in Example 8 (tested with 7β,27-dihydroxy-4-cholesten-3-one).

Example 8:

Effects of 7β,27-dihydroxy-4-cholesten-3-one on proliferation and viability of human malignant cells

Human colonic carcinoma (WiDr) and malignant melanoma (SKMEL-2) cell lines from American Type Culture Collection, U.S.A., and SV-40 virus transformed human fibroblasts (90-VA VI), a kind gift from dr. Stein (University of Colorado, Boulder, CO, U.S.A.), were grown in monolayers in tissue cultured flasks maintained in a 95% air/5% $CO_2$ atmosphere at 37°C in a humidified incubator and were cultured in Dulbecco's Minimal Eagle's Medium supplemented with essential and non-essential amino acids and 10% fetal calf serum (FCS, from Life Technologies, Inc., Stockholm, Sweden). Cells were seeded at a density of 5,000 cells per $cm^2$ in 9 $cm^2$ dishes in the media (2 ml) containing 10% FCS. The experiments were started 24-48 h later, at which time a cell density of approximately 10,000 per $cm^2$ had been reached (the cells were subconfluent also at the end of the incubations). All cells were then incubated for 24 h in the media (2 ml) lacking serum, before 7β,27-dihydroxy-4-cholesten-3-one (and 5-cholestene-3β,7β,27-triol for comparison) (tested at the concentration 2.5 μM) were added to the incubation media in freshly prepared ethanol solutions. The ethanol concentration of the media then became 1.0%. Control cells were incubated with the same volumes of ethanol but without sterols. The proliferation of cells was registered by counting in a light microscope the cell number in marked areas in the dishes after incubations for 24 h and 48 h, and cell death was recognized microscopically as detachment or lysis of cells in the monolayer cultures.

**TABLE X**

| Sterol structure[a] | Cell viability (%)[c] | | | | | |
|---|---|---|---|---|---|---|
| | Colonic carcinoma cells | | Malignant melanoma cells | | Transformed fibroblasts | |
| | 24 h[b] | 48 h[b] | 24 h[b] | 48 h[b] | 24 h[b] | 48 h[b] |
| None = Control | 126 | 87 | 122 | 113 | 123 | 95 |
| $C^5$-3β,7β,27-ol | 94 | 124 | 128 | 157 | 88 | 86 |
| $C^4$-7β,27-ol-3-one | 35 | 24 | 86 | 26 | 42 | 0 |

[a] C, cholestane; superscript indicates position of double bond; Greek letters denote configuration of hydroxyl groups.

[b] Incubation period

[c] Number of viable cells in relation to the number of cells at the beginning of the experiment (in %)

The results clearly show that the compounds of the invention are potent inducers of death in both human colonic and malignant melanoma cells. It should be pointed out that malignant melanoma is a form of cancer that is extremely resistant to chemotherapy.

Because of the high potency of these sterols and their selectivity in toxicity (affecting tumor-transformed human cells), they can be expected to be highly useful for treatment of various cancer diseases, in addition to those tested above.

Furthermore, because of their effects on cell growth one may expect that these sterols should also be useful for treatment of diseases caused by abnormally fast-growing non-tumor cells and psoriasis, particularly when the sterols can be applied locally.

Example 9

Synthesis of 7β,25-dihydroxy-4-cholesten-3-one and 7α,25-dihydroxy-4-cholesten-3-one

a) Preparation of 25-hydroxycholestrol acetate

The acetate is prepared from 2 mg dried commercially available 25-hydroxycholesterol (Sigma) to which 0.5 ml pyridine is added prior to ultrasound. 0.5 ml acetic anhydride is added and the mixture resubjected to ultrasound. The reaction mixture is allowed to stand at room temperature for 2.5 hours and the reaction is quenched with 5 ml $H_2O$ and allowed to stand for 15 minutes. 3 ml ethyl acetate is added and the mixture agitated and subjected to ultrasound and then centrifuged for several minutes. The aqueous phase (the bottom phase) is transferred to a fresh vessel and saved. 3 ml of $H_2O$ is added to the ethyl acetate phase and the mixture is shaken/ultrasounded/centrifuged and decanted to the vessel. This procedure is repeated to result in an aqueous phase of 3 x 3 ml.

b) Trimethylsilylation

The resulting ethyl acetate phase is rinsed with a small amount of ethyl acetate into a fresh vessel and blown dry. The trimethylsilyl ether (TMS) of the 25 hydroxy group is prepared by subjecting the product to 0.5 ml pyridine/hexamethyldisilane/trimethylchlorosilazane, 3:2:1 which is allowed to react at 60° for 30 minutes. The product is blown dry and dissolved in hexane, ultrasounded and transferred to a 25 ml Florence flask with a little hexane.

c) TBB-oxidation

The resulting product is subject to TBB-oxidation as follows:

The hexane phase is blown dry, dissolved in 1.5 ml concentrated acetic acid and ultrasounded. 1.5 mg copper II bromide, 10 μl TBB (tert-butylperbenzoate) and an agitating bead is added and the reaction proceeds under $N_2$ at 100°C for 5 minutes. The product is allowed to cool and is transferred to a separating funnel. The flask is

rinsed out into the funnel with 5 ml $H_2O$, then 40 ml hexane. The mixture is agitated and the aqueous phase discarded. 5 ml of 5% $NaHCO_3$ is added and the product agitated, while allowing for gas evolution. The bicarbonate phase is discarded. The latter step is repeated. The product is washed twice with 5 ml $H_2O$. Th pH should be neutral. The hexane phase is transferred to a Florence flask and blown dry.

d) Hydrolysis

The resulting product is hydrolysed as follows:
5 ml of 5% KOH in methanol is added, ultrsounded and the mixture is incubated for 1 hour at 50°C in a water bath with gentle agitation. The reaction is quenched with 5 ml $H_2O$ which is mixed and the product is neutralised to ca. pH 7 with concentrated acetic acid. The product is subject to a C18 column (ODS silica) (1.5 x 0.8 cm) rinsed with 10 ml $H_2O$. The product is eluted with 8 ml methanol, evaporated and dissolved in 1 ml methanol. 10μl is retained for GLC.

e) Purification

The resulting product is purified on a Unisil column (Clarkson Chem Co, Williamsport PA, USA) (mesh 200-325, 3 x 0.8 cm packed in hexane, washed with 5 ml hexane/ethyl acetate 1.1) after being evaporated and dissolved in 3 ml hexane/ethyl acetate 1:1. 11 fractions are collected as follows:

| | | |
|---|---|---|
| 1 | product (3 ml hexane/ethyl acetate 1:1) & additional 3 ml hexane/ethyl acetate 1:1 | |
| 2,3 | 2 x 3 ml hexane/ethyl acetate | 40:60 |
| 4,5 | 2 x 3 ml hexane/ethyl acetate | 30:70 |
| 6,7 | 2 x 3 ml hexane/ethyl acetate | 20:80 |
| 8,9 | 2 x 3 ml hexane/ethyl acetate | 10:90 |
| 10 | 6 ml ehtyl acetate | |
| 11 | 6 ml methanol | |

The flask is rinsed with all fractions, the fractions are evaporated and dissolved in 0.5 ml methanol. 20 μl is retained for GLC. The remainder is stored at -70°C

pending GLC analysis. Fractions 4 and 5 contain 5-cholestene-3β,7β,25 triol. Fractions 6, 7 & 8 contain 5-cholestene-3β,7α,25-triol.

f) Oxidation with cholesterol oxidase

The 3β-hydroxy group is oxidised and the 5-double bond isomerised) via cholesterol oxidase (C-1512, Sigma) as follows. Fractions 4 and 5 are dissolved in 0.5 ml isopropanol. 6 ml of 0.5 M phosphate buffer, pH 7.0 and 5 U cholesterol oxidase added. The mixture is incubated at 37°C for 3 hours with gentle agitation. The reaction is then quenched with 9 ml methanol and subjected to the C18 column, (1.5 x 0.8 cm). The eluent is pooled and evaporated until $H_2O$ remains (ca 6 ml). 1 ml methanol is added and after mixing the product is resubjected to the same C18 column. When the product has gone through, the column is rinsed with 10 ml $H_2O$ (2+2+6) and eluted with 8 ml methanol direct into a Florence flask. The product is evaporated, transferred to test tube with methanol, evaportated and dissolved in 0.5 ml ethanol. 20μl is retained for GLC and GS/MS.

g) HPLC Purification

The resulting 7α,25-diydroxy-4-cholesten-3-one and 7β,25-diydroxy-4-cholesten-3-one are then purified with reversed phase HPLC (column: LiChrosper, 250 x 4 mm, Hibar, 100 RP-18, 5 μ, Merck) in 85% methanol, UV detector at 240 nm, retention time 7.3 minutes for the 7β anomer and 7.8 minutes for the 7α anomer. The effluent is collected over a Florence flask, evaporated, transferred to a test tube with methanol, evaporated and dissolved in 0.5 ml ethanol. 20 μl is retained for GLC and GC/MS.

Example 10

Preparation of 7β,27-dihydroxy-4-cholesten-3-one

A Preparation of 27-hydroxycholesterol

a) A Clemmensen reduction of diosgenin is carried out with fresh zink amalgam prepared from 60 g zink filings, 4.5 g mercury II chloride, 3.0 ml conc. HCl and 75 ml $H_20$. The mixture is agitated in a multinecked round flask for 5 minutes and decanted. 200 ml ethanol and 1.2 g diosgenin (Sigma) is added and refluxed. Over 45 minutes 60 ml conc. HCl is added dropwise, followed by 15 min continued reflux. The mixture is allowed to cool to room temperature. 1.5 l ice-cold water is added dropwise and the reaction allowed to stand under refrigeration for around an hour. The water is then filtered away and the dry mass transferred to a Florence flask. 30 ml diethyl ether is added and the mixture stirred at room temperature for 1o minutes. The ether is filtered off and mass transferred to a small evaporating flask an dissolved in a minimum of ethyl acetate using a little warm water. Crystallisation is commenced firstly at room temperature and later overnight in the coolroom to afford tetrahydrodiosgenin (16β,27-dihydroxycholesterol).

b) Chromo-oxidation

The product is oxidised in 4 batches as follows. 20 mg chrome trioxide in 0.1 ml $H_2O$ and 0.2 ml acetic acid is added dropwise to a stirred mixture of 125 mg tetrahydrodiosgenin and 0.62 g NaAc in 22.5 ml glacial acetate. The reaction continues for 18 hours at 25 °C. A few drops of methanol are then added to destroy excess reagent. The mixture is diluted with 25 ml cold water and extracted with 40 ml $MeCl_2$. The methylene chloride phase is rinsed with around 10 ml $H_2O$ and then with 5% sodium bicarbonate and $H_2O$ until neutral by pH paper. The $MeCl_2$ is dried with a spoon of waterfree $Na_2SO_4$ and the $MeCl_2$ phase filtered down into a Florence flask and evaporated. A GLC sample retained. The remaining product is subjected to a silicon column 8 x 0.8 cm in 30% ethyl acetate/TMP (trimethylpentane) in one fifth aliquots. The fractionation comprises:

5 x 10 ml 30% ethyl acetate/TMP

10 x 5 ml 40% ethyl acetate/TMP.

Around 0.1% of each fraction is assayed by GLC and the fractions containing 16-keto-27-hydroxycholesterol are pooled (generally fractions 3-6 of the 40% blend).

c) Reduction of 16-keto-27 hydroxycholesterol

The resulting product from step b) (170 mg) is transferred to a small Florence flask and 0.11 g KOH in 3.5 ml triethylene glycol, 0.1 ml hydrazine and a couple of agitation beads are added. The mixture is refluxed for 15 minutes and then refrigerated for 1 hour. The mixture is dropwise added to 15 ml 0.5 M HCl, filtered and rinsed with ice cold water. The dry material is transferred to a small evaporating flask and recrystallised with ethyl acetate. Purity is assayed by GLC & GC/MS. Yield: 75 mg 27-hydroxycholesterol, 45 mg as crystals.

B Preparation of 7β,27-dihydroxy-4-cholesten-3-one

a) The acetate of the product of step A is prepared in the same manner as Example 9 step a), with the exception that the reaction was quenched with 5 ml $H_2O$. The TMS step b) of Example 9 is omitted.

b) The resulting ethyl acetate phase is blown dry and subject to $Na_2CrO_4$ oxidation by the addition of 500 μl concentrated acetic acid and 200 μl $Na_2CrO_4$. The reaction proceeds overnight at room temperature with a magnetic stirrer. The oxidation is stopped with 3ml of $H_2O$ added cautiously to the reaction vessel sitting in an ice bath in a ventilation hood. The mixture is allowed to stand for 15 minutes and is then neutralised to pH 7 with 1M NaOH.

c) The resulting product is blown dry and reduced with 3 mg $NaBH_4$ following after being dissolved in 1 ml dry ethanol (from a freshly opened bottle). The reaction proceeds in an ultrasound bath for 30 minutes and then around 1.5 hours at room temperature. The reaction is quenched with 100 ml acetone and 3 ml $H_2O$. The product is subjected to a rinsed C18 column with 5 ml $H_2O$. The product

is eluted with 8 ml methanol (2+2+4 ml)into a Florence flask. 50 µl is assayed via TMS and GLC.

d) The resulting product is evaporated dry and hydrolysed via the addition of 0.3 ml isopropanol and ultrasound, followed by the addition of 3 ml methanol. 259 ml of 12.5 M NaOH is added mixed and ultrasounded. The product is incubated at 50°C for 2 hours in a water bath. The reaction is stopped by blending in 6 ml $H_2O$. The product is neutralised to pH 7 with conc. acetic acid and then subjected to a rinsed C18 column. The 4 fractions comprise:

| | |
|---|---|
| 1 | product |
| 2 | 10 ml $H_2O$ via round flask & ultrasound (2+2+6 ml) |
| 3 | 5 ml 30% methanol (do.) |
| 4 | product is eluted with 10 ml methanol (do.) |

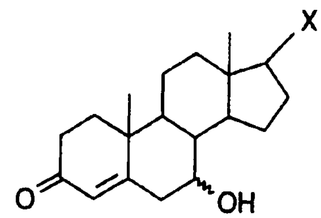
50 µl is assayed via TMS and GLC.

e) The remainder of the product is purified on a Unisil column as described in example 9 part d, wherein the 50 µl check samples are assayed byGLC after TMS treatment. Fraction 6 contains 5-cholestene-3β,7β,27 triol and fraction 8 contains 5-cholestene-3β,7α,27 triol.

e) Fraction 6 from step d) is oxidised by cholesterol oxidase as described in example 9, step e) and the resultant 7β,27-dihydroxy-4-cholesten-3-one purified by HPLC as described in example 9. Retention time 8.7 minutes.

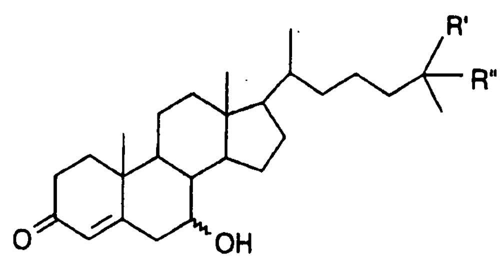
# CLAIMS

1. A compound of the formula I:




wherein X is a straight or branched, hydroxy substituted $C_1$-$C_{15}$ hydrocarbon chain, for use in medicine.

2. A compound according to claim 1 with the formula IA:




wherein one of R' and R"is OH and the other is H, for use in medicine.

3. The compound according to claim 2 denoted 7β,25-dihydroxy-4-cholesten-3-one, for use in medicine.

4. The compound according to claim 2 denoted 7α,25-dihydroxy-4-cholesten-3-one for use in medicine.

5. The compound according to claim 2 denoted 7β,27-dihydroxy-4-cholesten-3-one for use in medicine.

6. Use of a compound as defined in any one of claims 1 to 5 in the manufacture of a medicament for the treatment of conditions associated with rapidly growing cells, such as cancers, virus transformed mammalian cells or psoriasis.

7. Use according to claim 6 wherein the cancer comprises a sarcoma, such as soft tissue sarcoma, a myeloproliferative tumour, such as leukaemia, glioblastoma, pancreatic, ovarian, adenocystic, colonic or breast cancer

8. Use according to claim 6 wherein the cancer comprises melanoma..

9. The enantiomer 7β,25-dihydroxy-4-cholesten-3-one in substantially pure form.

10. A pharmaceutical composition for the treatment of conditions associated with rapidly growing cells, such as cancers, virus transformed mammalian cells or psoriasis, comprising a compound as defined in any one of claims 1 to 5 in admixture with a physiologically acceptable diluent or pharmaceutical carrier.

11. A pharmaceutical composition according to claim 9 wherein the cancer comprises a sarcoma, such as soft tissue sarcoma, a myeloproliferative tumour, such as leukaemia, glioblastoma, pancreatic, ovarian, adenocystic, colonic or breast cancer.

12. A pharmaceutical composition according to claim 9 wherein cancer comprises melanoma.

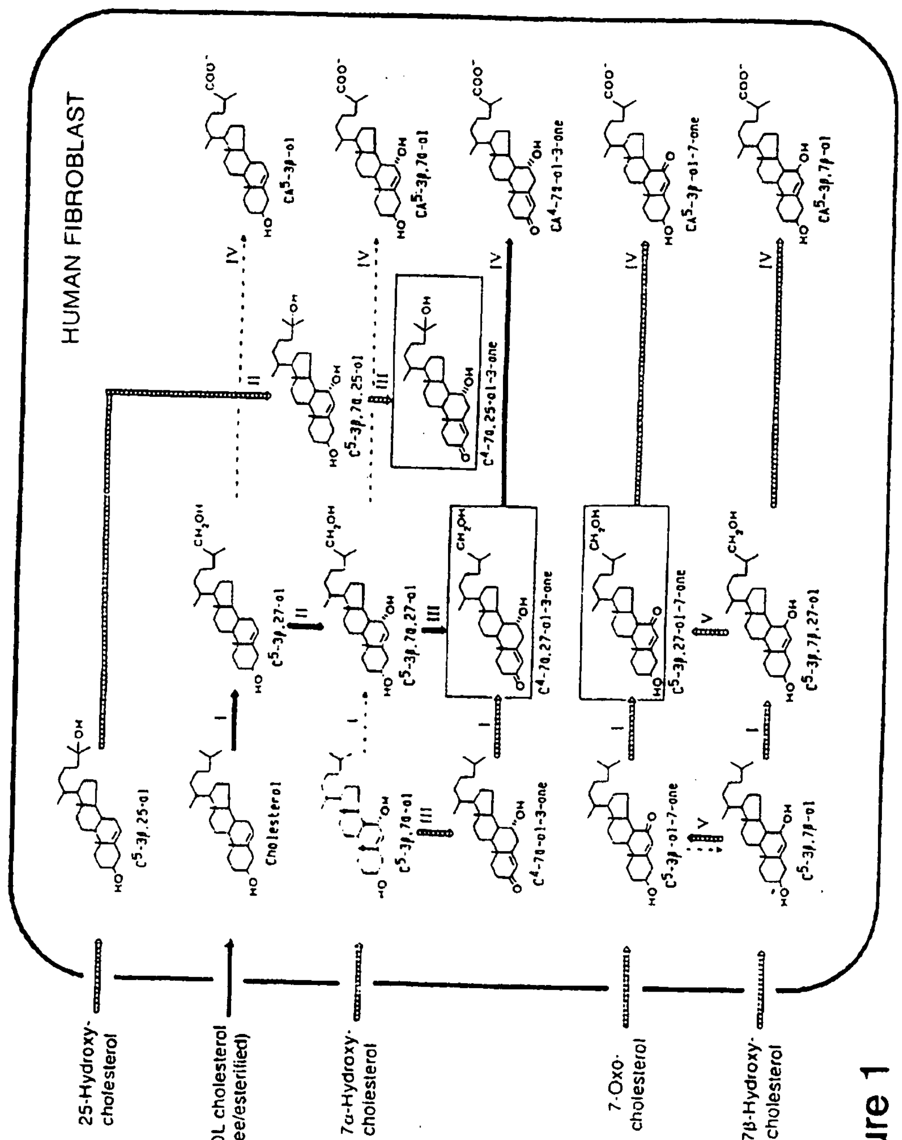
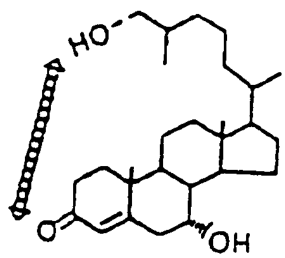
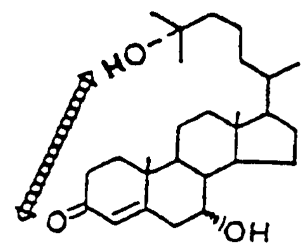

HUMAN FIBROBLAST
25-Hydroxy-cholesterol
LDL cholesterol (free/esterified)
7α-Hydroxy-cholesterol
7-Oxo-cholesterol
7β-Hydroxy-cholesterol

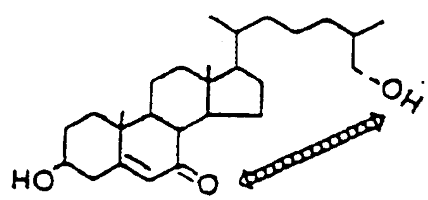
Figure 1



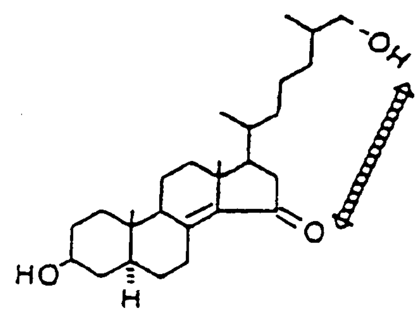
I



II



III



IV

Figure 2

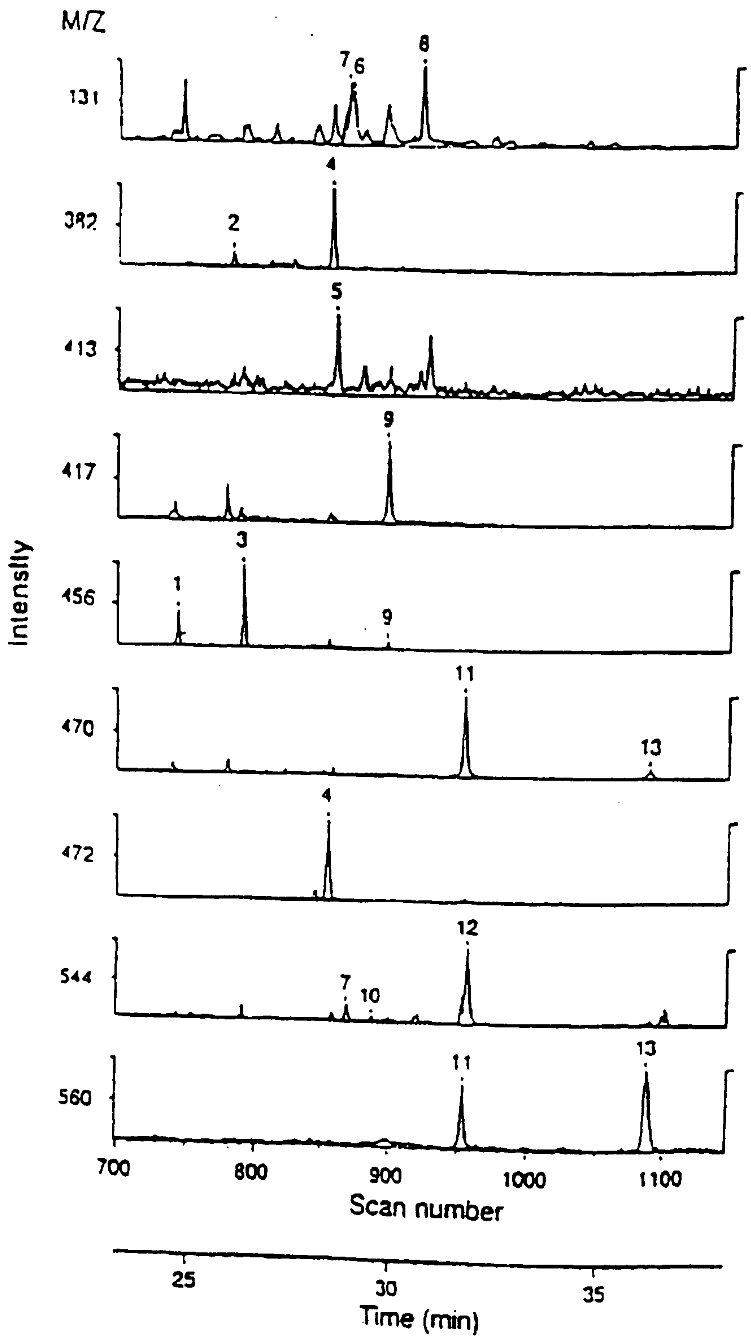

M/Z
131
382
413
417
456
470
472
544
560
Intensity
7
6
8
4
2
5
9
3
1
9
11
13
4
12
7
10
11
13
700
800
900
1000
1100
Scan number
25
30
35
Time (min)

Figure 3

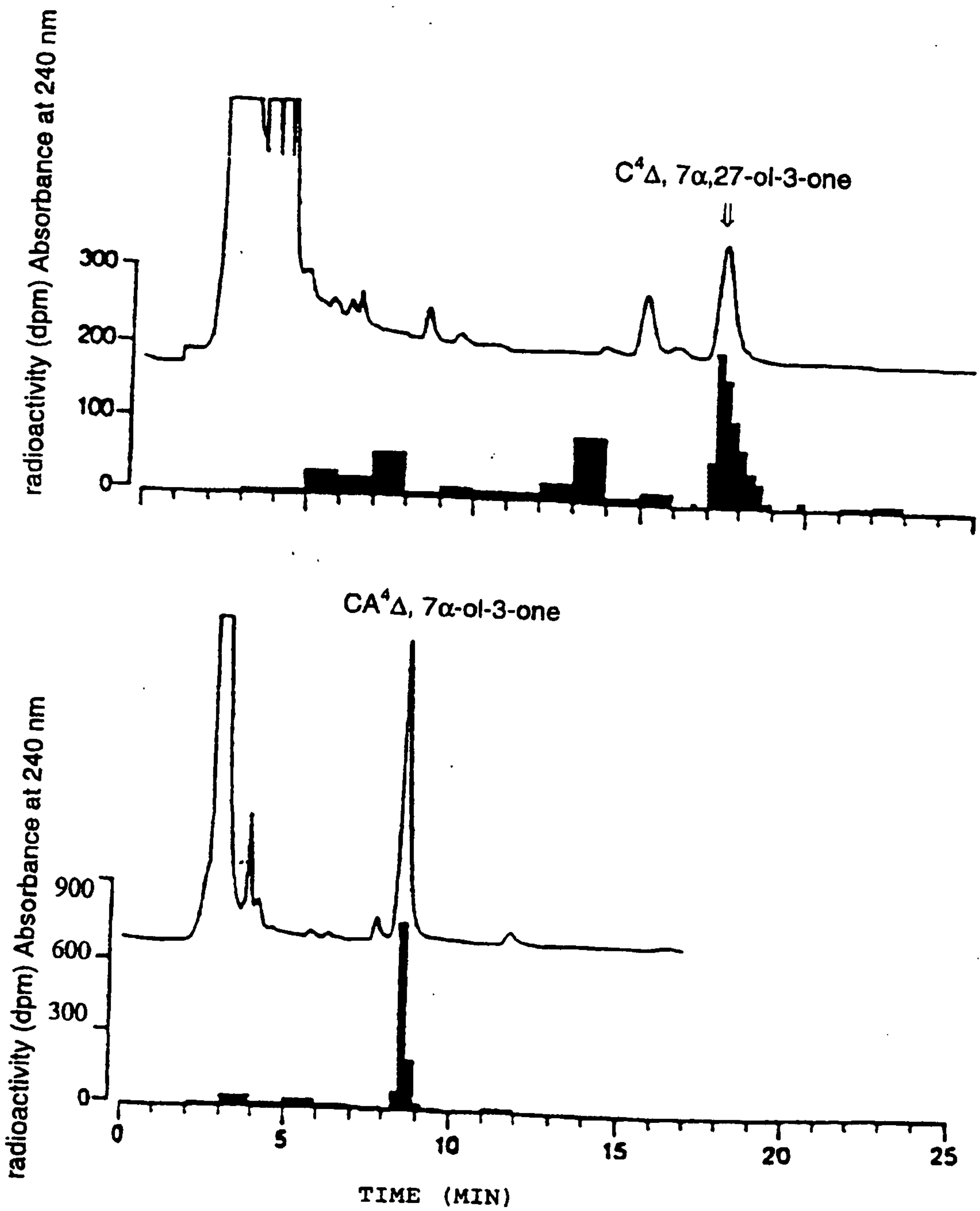

radioactivity (dpm) Absorbance at 240 nm
300
200
100
0
C⁴Δ, 7α,27-ol-3-one
CA⁴Δ, 7α-ol-3-one
radioactivity (dpm) Absorbance at 240 nm
900
600
300
0
0
5
10
15
20
25
TIME (MIN)

Figure 4

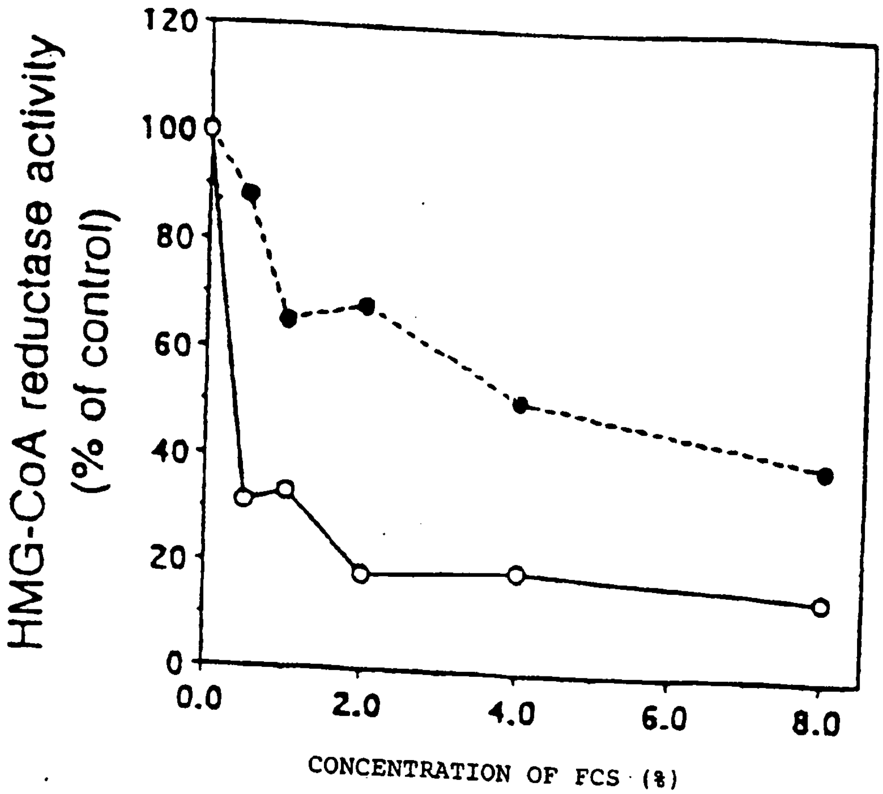

HMG-CoA reductase activity
(% of control)
120
100
80
60
40
20
0
0.0
2.0
4.0
6.0
8.0
CONCENTRATION OF FCS (%)

Figure 5

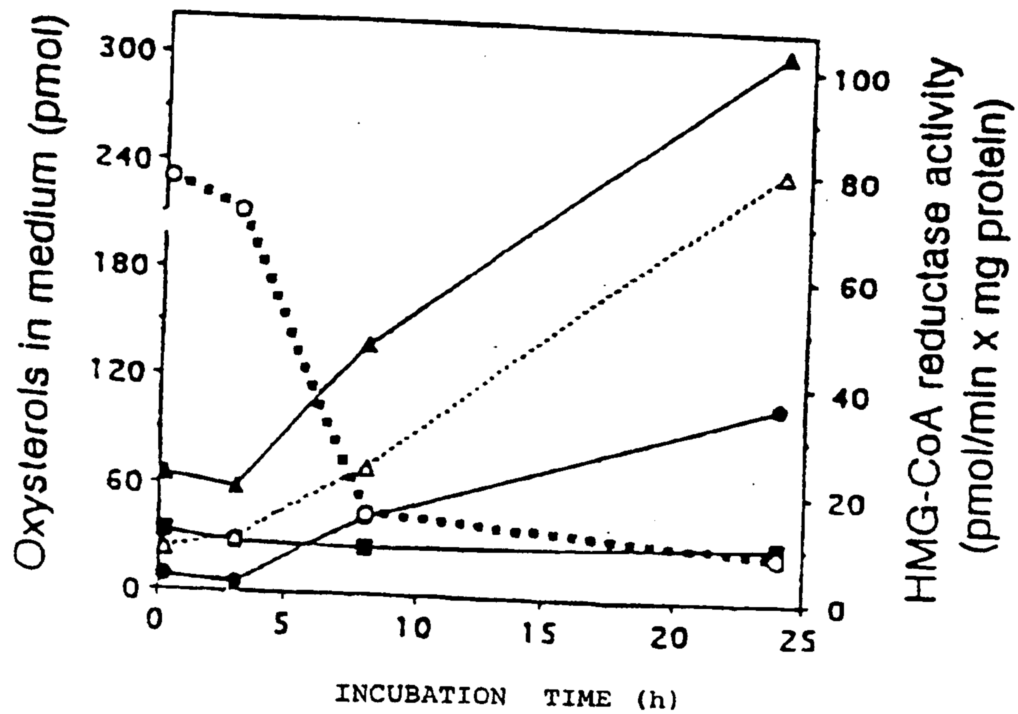

Oxysterols in medium (pmol)
300
240
180
120
60
0
HMG-CoA reductase activity
(pmol/min x mg protein)
100
80
60
40
20
0
0
5
10
15
20
25
INCUBATION TIME (h)

Figure 6

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/SE 97/00936

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C07J9/00 A61K31/575

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07J A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | L. SWELL ET AL: "An in vivo evaluation of the quantitative significance of several potential pathways to cholic and chenodeoxycholic acids from cholesterol in man" JOURNAL OF LIPID RESEARCH, vol. 21, no. 4, 1980, pages 455-466, XP002043822 see the whole document --- | 1,2, 10-12 |
| Y | D. PAYNE ET AL: "A Novel Nonhepatic Hydroxycholesterol 7.alpha.-Hydroxylase That is Markedly Stimulated by Interleukin-1.beta." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 32, 11 August 1995, MD US, pages 18888-18896, XP002043823 see page 18889, column 1, line 1 - line 3 --- | 1,2,4, 6-8, 10-12 |

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search
16 October 1997

Date of mailing of the international search report
05.11.97

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Authorized officer
Watchorn, P

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/SE 97/00936

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | CHEMICAL ABSTRACTS, vol. 122, no. 13, 27 March 1995 Columbus, Ohio, US; abstract no. 157013, M. AXELSON ET AL: "Structural Specificity in The Suppression of HMG-CoA Reductase in Human Fibroblasts by Intermediates in Bile Acid Synthesis" page 646; column 1; XP002043825 see abstract & JOURNAL OF LIPID RESEARCH, vol. 36, no. 2, 1995, pages 290-298, --- | 1,2,4, 6-8, 10-12 |
| P,X | M. AXELSON ET AL: "27-Hydroxylated Low Density Lipoprotein (LDL) Cholesterol Can Be Converted to 7.alpha.,27-Dihydroxy-4-cholesten-3-one (Cytosterone) before Suppressing Cholesterol Production in Normal Fibroblasts" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 22, 31 May 1996, MD US, pages 12724-12736, XP002043824 see page 12731, column 1, paragraph 2 see page 12731, column 2, paragraph 3 --- | 1-12 |
| P,X | CHEMICAL ABSTRACTS, vol. 126, no. 13, 31 March 1997 Columbus, Ohio, US; abstract no. 169459, J. ZHANG ET AL: "Studies on The Relationship between 7.alpha.-Hydroxylation and the Ability of 25- and 27-Hydroxylcholesterol to Suppress the Activity of HMG-CoA Reductase" page 396; column 2; XP002043826 see abstract & BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1344, no. 3, 1997, pages 241-249, ----- | 1-12 |

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